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# THE ACTION OF STRYCHNINE AND NICOTINE ON THE NEUROMUSCULAR MECHANISM OF ASTERIAS.

By A. R. MOORE.

*(From the Physiological Laboratory of Rutgers College, New Brunswick, N. J., and the Marine Biological Laboratory, Woods Hole, Mass.)*

(Received for publication, October 23, 1919.)

It has been demonstrated that strychninization in *Asterias* is followed by extreme dorsal flexure of the rays and consequent inability to make righting movements[1] (Figs. 1, 2, and 3). This "spasm" position of the rays is accelerated and accentuated by sensory stimulation. In order to determine the location of the muscles which cause this action, the dorsal myodermal sheath is cut from all five rays of a specimen of *Asterias*. This leaves only the ventral floors of the rays attached to the central disk. When such a preparation is put into a solution of strychnine in sea water, the dorsal flexure of the rays takes place, although not so completely as in the intact animal. This result shows that the ventral walls of the rays contain muscles which act to bend the floor dorsally. The excised pieces of dorsal myodermal sheath when strychninized show dorsal flexure. Therefore, it is evident that in the intact animal, muscles of both the top sheath and floor of the ray act together to flex the ray dorsally.

The reverse picture for the entire animal may be obtained by immersing small specimens of *Asterias* in a solution of alkaloidal nicotine in sea water, concentration 1:50,000. Within a few minutes the rays bend downward at the tips and the central disk is raised (Fig. 4), while at the same time the tube feet are completely shortened and withdrawn. The ventral flexure increases in degree until the animal is a compact mass (Fig. 5). At this point the starfish falls over on its side, where it remains in the knotted condition shown, for 15 or 20 minutes, after which extension takes place.

[1] Moore, A. R., *J. Gen. Physiol.*, 1918–19, i, 97.

Severed rays, when nicotinized, show ventral flexure. Likewise the excised floors of the rays in nicotine solution flex ventrally so far as to form half circles. The isolated top sheaths of the rays, however,

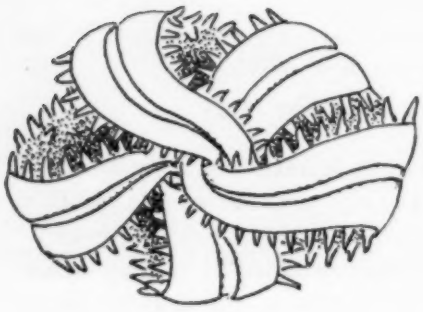

FIG. 1.

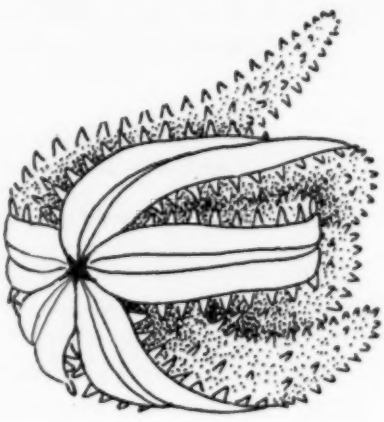

FIG. 2.

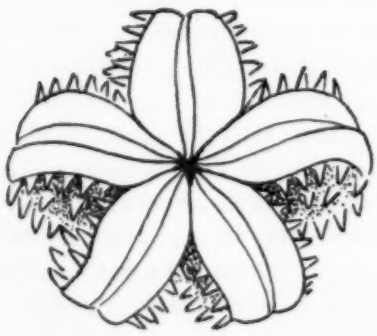

FIG. 3.

FIGS. 1, 2, and 3. Positions taken as the result of strychninization. The arms are bent dorsally to such an extent that they cover up almost completely the back of the animal, a condition illustrated in Figs. 1 and 2. Fig. 3 shows an inverted animal unable to right itself, since all the arms bend dorsally.

when nicotinized, bend dorsally just as if strychninized. From these facts it must be concluded that ventral flexure of the ray depends solely upon certain muscles in the floor of the ray, and that these are

capable of more powerful action than the muscles of the top sheath. It is also clear from the results of strychninization and nicotinization

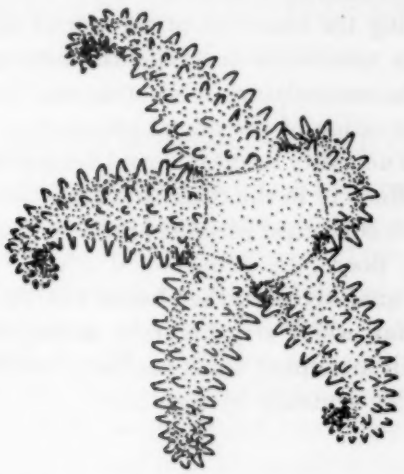

FIG. 4. Initial stage in nicotinization. The effect of the nicotine is the reverse from that of strychnine, since the arms bend toward the ventral side and raise the central disk from the floor.

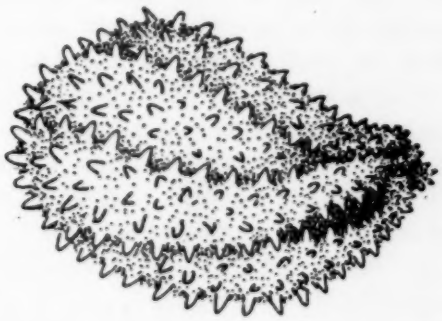

FIG. 5. Final position resulting from nicotinization. The arms are bent towards the ventral side to such an extent that the ventral surface of the animal is no longer seen; the animal falls on its side.

that the ventral floor of the ray contains muscle groups which act antagonistically. If we assume both these drugs to act upon the nervous elements controlling this musculature, we may regard strych-

nine as an excitor of all such elements. Dorsal flexure then occurs because the sum of the muscles causing it is greater and more powerful than that of the musculature acting ventrally. Nicotine, on the other hand, while exciting the elements of the dorsal sheath, as regards the ray floor, acts specifically to excite the nervous elements controlling the ventral musculature. Nicotine can have no excitatory effect on the innervation of the dorsal antagonists in the ray floor, since this would of necessity result in dorsal flexure of the ray. These facts indicate a difference in the chemical constitution of the two sets of nervous elements concerned with the functioning of the antagonistic muscles of the ray floor.

The preceding analysis is further borne out by the fact that a nicotinized *Asterias* when strychninized shows the characteristic dorsal flexure, while an animal which has been first strychninized cannot be caused to flex ventrally by nicotine.

# THE CHROMOSOMES OF PARTHENOGENETIC FROGS.

BY CHARLES L. PARMENTER.

*(From the Zoological Laboratory of the University of Pennsylvania, Philadelphia.)*

(Received for publication, December 1, 1919.)

Among the important features of interest in connection with parthenogenetic frogs are the chromosome number and the sex-determining mechanism. At the suggestion of Doctor Loeb, I have undertaken the investigation of these problems by using some of the parthenogenetic frogs and tadpoles which he has thus far raised.

Previous to 1919, Doctor Loeb had succeeded in raising twenty frogs to the adult condition.[1] Fifteen of these were males, three were females, and the sex of the remaining two was undetermined. In 1919, he succeeded in raising 65 tadpoles to metamorphosis. One of these has metamorphosed, seventeen have been fixed for cytological purposes, five have died, and the rest are still tadpoles.

The chromosomes of the gonads of one of these adult males and of thirteen of the tadpoles have been examined. In all these individuals the number is clearly diploid. The only two spermatogonial complexes of the adult male, sufficiently clear for study, show about twenty chromosomes distinctly and others superimposed, as previously stated.[1] Among the cells undergoing maturation are tetrads in the late prophase stage. These tetrads appear as rings, either completely closed or slightly open at one point. They are apparently of the same form as tetrads of the normal material. Their number is clearly haploid, but an exact count has not yet been obtained. In the sections of the gonads of the thirteen tadpoles there are many complexes in which all but one or two chromosomes are entirely clear, and several mitoses in which all the chromosomes are well separated but cannot be counted with certainty because the cell has been cut in sectioning. However, the number of chromosomes in a limited number of complexes of two individuals is definitely twenty-six.

[1] Loeb, J., *Proc. Nat. Acad. Sc.*, 1918, iv, 60.

Since none of the individuals studied has the haploid number, it is probable that the diploid number is characteristic for the majority, if not for all the parthenogenetic individuals. The diploid number, as well as the similarity in form of the tetrads of the parthenogenetic and normal animals, may have been brought about by the retention of the second polar body, or by a premature division of the chromosomes without the division of the cell body just before the first cleavage. It is hoped in the near future to determine how this condition has arisen.

At the present time the mechanism producing the two sexes in both the normal and parthenogenetic frogs is undetermined. Levy[2] in *Rana esculenta*, and Swingle[3] in *Rana pipiens*, describe a sex chromosome in the normal male. But the evidence of neither of these authors is convincing. There are some interesting theoretical possibilities by which a predominance of parthenogenetic males over females, as indicated by the numbers so far obtained, might be produced, and it is hoped that further observations will reveal the exact mechanism.

[2] Levy, F., *Arch. Mikr. Anat., 2te Abt.*, 1915, lxxxvi, 85.
[3] Swingle, W. W., *Biol. Bull.*, 1917, xxxiii, 70.

# STUDIES ON BIOLUMINESCENCE.

## XII. The Action of Acid and of Light in the Reduction of Cypridina Oxyluciferin.

By E. NEWTON HARVEY.

*(From the Physiological Laboratory, Princeton University, Princeton.)*

(Received for publication, October 17, 1919.)

In the luminous crustacean, *Cypridina*, a substance, luciferin, is oxidized in the presence of water, oxygen, and another substance, luciferase, with the production of light. I have called the oxidation product of luciferin oxyluciferin; it is obtained by allowing luciferin in solution to oxidize completely. The oxidation is accelerated at high temperatures. An extract of *Cypridina*, boiled until it no longer gives light on mixing with luciferase, will be spoken of throughout this paper as an oxyluciferin solution (Harvey, 1918–19). A solution of oxyluciferin has the same reaction as a luciferin solution. It is slightly alkaline, about pH = 9.

No measurable production of $CO_2$ (Harvey, 1919–20, *a*) or of heat (Harvey, 1919–20, *b*) accompanies the oxidation of luciferin. The reaction, luciferin → oxyluciferin, therefore involves a relatively slight energy change and should be readily reversible. An oxyluciferin solution, however, will not reduce to luciferin spontaneously on standing (in the dark) or on removal, with an air pump, of the dissolved oxygen. However, if we add to an oxyluciferin solution some milk, frog muscle suspension, yeast cells, bacteria, or Mg powder plus acid, which results in the evolution of nascent hydrogen, luciferin will be formed and can be tested by its ability to luminesce with luciferase. As the reducing action of milk, tissues, bacteria, and nascent hydrogen are so well known, it seemed probable that they formed luciferin by removal of oxygen from, or addition of $H_2$ to, oxyluciferin, reducing it in the same way that methylene blue can be reduced (decolorized) by tissue cells, bacteria, nascent hydrogen, etc.

In my previous paper (1918–19) I called attention to the fact that

acid also plays a part in the formation of luciferin from oxyluciferin. "If one saturates an oxyluciferin solution with $CO_2$ or adds a little dilute acetic acid and allows the solution to stand for 24 hours, a certain amount of reduction will occur" (page 143). Indeed it is not necessary to let the solution stand 24 hours. Luciferin will be formed from an oxyluciferin solution immediately on adding a little acid to it. HCl, $HNO_3$, or $H_2SO_4$ may also be used. The action begins when the solution of oxyluciferin, ordinarily slightly alkaline (pH = 9), is made neutral (pH = 7.1), as indicated in Table I.

TABLE I.

*Effect of Acid on Reduction of Oxyluciferin.*

| Solution. | pH | Luminescence with luciferase. | Remarks. |
|---|---|---|---|
| 20 cc. of oxyluciferin alone. | 9.01 | Negative. | |
| 20 cc. of oxyluciferin + 0.05 cc. of 5 per cent acetic acid. | 8.8 | " | |
| 20 cc. of oxyluciferin + 0.15 cc. of 5 per cent acetic acid. | 7.1 | Fair. | |
| 20 cc. of oxyluciferin + 0.03 cc. of 5 per cent acetic acid. | 5.9 | Good. | Acid forms precipitate in this oxyluciferin solution. |
| 20 cc. of oxyluciferin + 0.05 cc. of 5 per cent acetic acid. | | " | Acid forms precipitate in this oxyluciferin solution. |
| 20 cc. of oxyluciferin + 0.75 cc. of 5 per cent acetic acid. | | " * | Acid forms precipitate in this oxyluciferin solution. |

* Light disappears quickly because of the effect of the acidity on the luciferase.

The acid must act on the oxyluciferin as no luciferase or other enzymes destroyed on boiling are present.

The addition of acid, then, to a boiled extract of *Cypridina* (oxyluciferin) causes the formation of a substance (luciferin) which will give light with luciferase. It is possible that the action of bacteria (which produce $CO_2$), muscle tissue (which contains lactic acid), or Mg plus acid in forming luciferin is not the result of their reducing power but of their acidity. Fortunately we can test this matter by

the use of reducing fluids which are not acid. If they also form luciferin from oxyluciferin, a reduction must occur. Nascent H can be generated by the action of NaOH on Al or when finely divided Mg, Zn, or Al is placed in water. With Mg the water becomes only slightly alkaline from the formation of almost insoluble Mg $(OH)_2$. If we add some Al powder and dilute NaOH to an oxyluciferin solution, H is given off and luciferin is formed. As oxyluciferin cannot be formed by the addition of alkali alone we must have in this experiment a reduction of oxyluciferin in alkaline medium by the nascent H produced. Luciferin can also be formed by merely adding Al, Zn, or Mg dust to an oxyluciferin solution. Methylene blue can be readily reduced to its leuco base by Zn dust or Al plus NaOH

Indeed, if one adds some Al, Zn, or Mg powder to a solution of luciferase, light will appear whenever the solution is shaken. Luciferase solution must always contain the oxidation product of luciferin, oxyluciferin. In the presence of nascent H this is reduced to luciferin and, since the reaction of the medium is alkaline and luciferase is present, this is oxidized with light production, when, by shaking, air is dissolved. The light can never become very bright except at the surface because of the deficiency of oxygen in the solution. It would seem, then, that the action of bacteria, yeast, muscle cells, etc. on oxyluciferin must be due not entirely to their acid reaction but to their reducing power as well.

The above experiment is striking and instructive. To a test-tube of luciferase solution containing as it does oxyluciferin add some Zn dust or Mg powder, and the evolution of hydrogen begins. Conditions are now favorable for the reduction of oxyluciferin, and this occurs. Shake the contents of the tube to dissolve oxygen and light appears. Allow the tube to stand and the light soon disappears. Shake again and the light reappears. The reduction and oxidation processes can be demonstrated many times.

A similar experiment can be performed with luciferase plus oxyluciferin solution by the addition of $(NH_4)_2S$. This will serve also as another example of the reduction of oxyluciferin in an alkaline medium. Whenever we shake a tube of luciferase, oxyluciferin, and $(NH_4)_2S$, light will appear. When the tube is at rest it becomes dark. Even the merest touch is sufficient to agitate the tube con-

tents, and cause the solution of oxygen and the appearance of light. It is just as if we stimulated the tube to produce light and I believe the phenomenon has a deeper significance and a more fundamental similarity to the phenomena of stimulation than may at first appear. What more simple means of controlling a process can we think of than by admission or withdrawal of oxygen? The firefly turns on its light by stimulation, through nerves, of the luminous organ. *Noctiluca* flashes on stimulation of any kind, even the slightest agitation causing a brilliant emission of light. If the stimulation process means merely the admission of oxygen to the photogenic cells, we have a mechanism in the cell itself for automatically producing the light. The admission of oxygen results in aerobic conditions, and luciferin in the presence of luciferase can then oxidize to oxyluciferin with luminescence. When the oxygen is used up, the light ceases, anaerobic conditions prevail, and the oxyluciferin is reduced to luciferin again. Thus, luciferin is reformed during the rest period of *Noctiluca* or between the flashes of the firefly. What more efficient type of light than this is to be desired.

Again, methylene blue offers an interesting parallel to oxyluciferin. A little $(NH_4)_2S$ added to a methylene blue solution will reduce (decolorize) it to the leuco base. If the tube is now shaken, the blue color returns. On standing reduction again occurs. The process can be repeated a number of times, the reaction going in one or the other direction depending on the oxygen content of the mixture.

As methylene blue contains no oxygen its reduction consists in the addition of two atoms of hydrogen. When leucomethylene blue oxidizes, water is formed by the union of these two atoms of hydrogen with oxygen, thus

$$\underset{\text{(Leucomethylene blue)}}{C_{16}H_{20}N_3SCl} + O \rightleftarrows \underset{\text{(Methylene blue)}}{C_{16}H_{18}N_3SCl} + H_2O$$

More briefly

$$MH_2 + O \rightleftarrows M + H_2O$$

To reduce methylene blue we can add the two hydrogen atoms directly from nascent hydrogen formed in the solution or we can split up water by a catalyzer in the presence of some substance which will take up the oxygen of water. The hydrogen of water then adds to the methylene blue, thus

$$NaH_2PO_2 + H_2O + Pd = NaH_2PO_3 + H_2 + Pd$$
(Sodium hypophosphite) (Sodium phosphite)

This reaction occurs in the presence of finely divided palladium. The methylene blue is reduced by the $H_2$, and the hypophosphite is oxidized. Since oxyluciferin can be reduced by palladium and sodium hypophosphite (Harvey 1918–19), it is probable that we can write the equation for the reduction of oxyluciferin and oxidation of luciferin in a similar manner to that of methylene blue.

$$\text{Luciferin} + O \rightleftarrows \text{oxyluciferin} + H_2O$$

More briefly

$$LH_2 + O \rightleftarrows L + H_2O$$

Just as in the case of methylene blue the reaction proceeds in the right hand direction spontaneously if the pressure of O is sufficiently high. If luciferase is also present we have luminescence.

$$LH_2 + O + \text{luciferase} \rightleftarrows L + H_2O + \text{luciferase (luminescence)}$$

The reaction proceeds in the left hand direction under low oxygen pressure, in the presence of nascent hydrogen, or with some catalyzer which is able to split water, transferring the $H_2$ to oxyluciferin and the O to an acceptor (A). $NaH_2PO_2$ plays the part of the acceptor.

$$L + H_2O + A + Pd \rightleftarrows LH_2 + AO + Pd$$

This appears to be the way in which the reducing enzymes or perhydridases (comparable to the Pd) of living tissues act (Bach, 1911, 1912, 1913), and the action of yeast cells, bacteria, muscle suspensions, etc. in reducing oxyluciferin must occur in the same manner.

If we assume that the $LH_2$ (luciferin) compound is dissociated even to the slightest extent into L and hydrogen, the hydrogen ion will shift the equilibrium toward the formation of that substance which involves the taking up of hydrogen. Consequently we may obtain a partial formation of luciferin by adding an acid to oxyluciferin. Reduction of the hydrogen ion concentration tends to shift the equilibrium in the opposite direction. Consequently, addition of alkali favors the oxidation of luciferin and it is quite generally true that biological oxidations are favored by an alkaline reaction. In addi-

tion oxygen in alkaline medium has a higher oxidation potential than in neutral or acid media. I believe this is the explanation of the action of acid in the formation of luciferin from oxyluciferin.

Addition of acid is not the only means of favoring the formation of luciferin from oxyluciferin. Any reaction which proceeds in one direction with evolution of light should, theoretically, proceed in the opposite direction under the influence of light. As far as I know the case of a reaction, photogenic in one direction and photochemical in the other direction, has never been described, unless we are to accept the cases of phosphorescence; for instance, the absorption of light by CaS and its emission in the dark. However, the reaction which occurs during phosphorescence cannot be stated.

It is a fact that light will cause the reduction of oxyluciferin. A tube of oxyluciferin exposed to sunlight for 6 hours or the mercury arc for 2 hours will be partially converted into luciferin. It will luminesce when luciferase is added while a control tube kept in darkness shows no trace of luciferin. The action is more marked with the ultra-violet, as a solution of oxyluciferin in a quartz tube showed more reduction than one in a glass tube when exposed for the same length of time to the quartz mercury arc. The reduction is not dependent on the formation of acid under the influence of light since two tubes of oxyluciferin, one kept in darkness and the other exposed to sunlight for 6 hours, had the same reaction, pH = 9.3. Of course some reducing substance might be formed under the influence of light but this is not very probable.

We may therefore write the reaction for luminescence in the following way.

$$\text{Luciferin } (LH_2) + O \underset{\text{Perhydridase.}}{\overset{\text{Darkness.}\ \text{Alkali.}\ \text{Luciferase.}}{\rightleftarrows}} \text{oxyluciferin } (L)^* + H_2O \text{ (luminescence)}$$
$$\text{Acid.}\ \text{Light.}$$

* It is unfortunate that Dubois (1918) has used the word oxyluciferin in quite a different sense from my own. His idea of the processes involved is as follows (p. 319):

As I stated in my previous paper (Harvey, 1918–19, page 144), "Acid thus favors reduction and hinders oxidation, while alkali favors oxidation and hinders reduction . . . . " so we may now add, light favors reduction and darkness favors oxidation of the luciferin ⇄ oxyluciferin reaction.

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Dubois, R., *Compt. rend. Soc. biol.*, 1918, lxxxi, 317; 1919, lxxxii, 840.

Harvey, E. N., *J. Gen. Physiol.*, 1918–19, i, 133; 1919–20, ii, *a*, 133; *b*, 137.

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"Coluciférase + préluciférine = luciférine;
Luciférase + luciférine = oxyluciférine;
Oxyluciférine + oxygène = lumière."

Coluciferase forms luciferin from a precursor, preluciferine, and Byla's peptone, lecithin, esculin, taurine, tyrosine, and asparagine will also form luciferin if mixed with coluciferase. We may perhaps add that luciferin must have an extraordinary chemical structure, if it can be formed from such diverse substances as lecithin, esculin, and taurine. My own view of the processes involved in luminescence is given in the text.

In a later paper, appearing after my own (Harvey, 1918–19), Dubois (1919) makes no mention of preluciferine and accepts in the case of *Pholas* my view that luciferin can be again formed from a boiled extract of luminous animals by reduction. The enzyme of *Pholas* concerned in this reduction ("a hydrogenase") can also liberate hydrogen from cane sugar. As this is Dubois's first mention of luciferin formation by reduction, I fail to see how, as Dubois states, I "rediscovered" this fact for *Cypridina*.

# STUDIES ON ENZYME ACTION.

## XVIII. The Saccharogenic Actions of Potato Juice.

By GRACE McGUIRE and K. GEORGE FALK.

*(From the Harriman Research Laboratory, the Roosevelt Hospital, New York.)*

(Received for publication, November 14, 1919.)

### INTRODUCTION.

The results of enzyme studies on a number of fresh and dehydrated vegetables were communicated in a previous paper.[1] In connection with the experiments on amylase, it was there stated that: "Potato juice, when tested for amylase by the method used with the other vegetable juices, gave an end-point which was so uncertain and difficult to determine that a different method had to be employed for it." In this paper are presented the results of a more careful study of potato amylase. Evidence is given showing the occurrence of a sucrase, and of a certain amount of sucrose or raffinose in potato juice.

### *Experimental Method.*

An extended study of potato amylase was published by Doby.[2] Attempts were made to repeat his work, but it was soon found that his observations were incomplete. In his work a series of tubes containing varying amounts of potato juice and Lintner soluble starch were set up for each experiment. After incubation, the supernatant liquid in each tube was pipetted from a residue, which separated as a rule, and was tested with iodine. The measure of the action was given by the tube in which no starch test was given with the iodine. Doby stated that the residue or precipitate contained no starch, but he did not present any experiments of his own in this connection. This statement apparently is based upon the work of

[1] Falk, K. G., McGuire, G., and Blount, E., *J. Biol. Chem.*, 1919, xxxviii, 229.
[2] Doby, G., *Biochem. Z.*, 1914, lxvii, 166.

other investigators,[3] who, however, worked with animal liquids, and not with filtered vegetable juices.

Upon repeating these experiments, it was found that, in accordance with the results of Doby, the supernatant liquids in many cases gave no test for starch, but on testing the precipitates with iodine, blue colors or compounds were obtained, showing the presence of starch. The presence of starch could also be shown by testing the whole mixture with iodine and shaking. A blue or purple color would be formed throughout the mixture, which would settle with the precipitate, leaving the supernatant liquid colorless. Wherever, therefore, a precipitate was formed, the results of the test as given by Doby must be looked upon as inconclusive. He found that sodium fluoride accelerated the amylase action. This observation can evidently be interpreted as the formation of a precipitate of the starch with the amylase, protein, or other material present which was produced more readily in the presence of this salt. With larger amounts of potato juice, hydrolysis of the starch would be complete, but the whole mixture must be tested in every case. These conditions were not realized by Doby in many of his experiments.

A different difficulty was encountered in attempting to use the Wohlgemuth[4] method for following amylase actions. In most of the experiments, in testing the final mixture or even the initial solution with iodine, an intense blue or purple color was obtained, which, however, faded rapidly, sometimes in a few seconds, leaving the mixture colorless. The rapidity of fading depended upon the quantity of potato juice present. It was necessary to add considerable iodine solution before a color, constant even for 3 or 4 minutes, could be obtained. Also, the end-points of these solutions were uncertain. Even when constant colors were obtained, these ranged from yellow-gray through gray to blue so that it was difficult to decide which tube represented the complete or final decomposition of the starch. Because of the presence of the chemically unknown, iodine-combining components of potato juice and the uncertainty of determining the

[3] Starkenstein, E., *Biochem. Z.*, 1910, xxiv, 191. Schirokauer, H., and Wilenko, G. G., *Biochem. Z.*, 1911, xxxiii, 275.

[4] Wohlgemuth, J., *Biochem. Z.*, 1908, ix, 1.

end-point of the actions, it was decided not to use this method in this work.

The Lintner[5] method, which was used with the vegetable juices for which the results were given in the former paper,[1] could not be used because the cuprous oxide was formed in such a finely divided state that it was impossible to tell whether the remaining Fehling's solution was still blue or had become colorless.

The method developed by Sherman, Kendall, and Clark,[6] precipitating and weighing the cuprous oxide from an excess of Fehling's solution under definite conditions, was finally adopted.

The general method of experimentation was as follows: The potatoes were thinly peeled, weighed, ground in a food chopper, squeezed through muslin, then the juice was filtered through fresh muslin, and finally filtered through asbestos on a Buchner funnel. The insoluble starch was removed as completely as possible in this way, but the solutions still contained a certain amount of soluble starch. The actions of the enzymes on the soluble starch and simpler carbohydrates present in the solutions, as well as on added starch and other substances, were followed. The tests were set up with 25 cc. of potato juice and 50 cc. of 1 per cent Lintner soluble starch solution, 50 cc. of water, or 50 cc. of 1 per cent cane sugar or maltose solution, as desired. Toluene was added as a preservative. Immediately after mixing, 5 to 15 cc. were pipetted out for the determination of the reducing action for blanks. The remainder was incubated for 18 to 24 hours at 37–40°C., and portions were then removed for tests. For the determination of the reducing action, Sherman's method was followed closely. The portion taken, 5 to 15 cc., was diluted in an Erlenmeyer flask with boiling water to 50 cc., 50 cc. of Fehling's solution were added, heated in a boiling water bath for 15 minutes, filtered on an asbestos mat in a porcelain Gooch crucible, washed with boiling water, alcohol, and ether, dried at 100°, and weighed. The cuprous oxide was then dissolved and the crucibles reweighed to check up possible mechanical losses of the mats. The starch solution was prepared by adding a paste of the Lintner soluble

[5] Lintner, C. J., *J. prakt. Chem.*, 1886, xxxiv, 378.

[6] Sherman, H. C., Kendall, E. C., and Clark, E. D., *J. Am. Chem. Soc.*, 1910, xxxii, 1083.

starch in water to the requisite amount of boiling water, boiling for 1 to 2 minutes, and cooling rapidly. The added sugars which were used (sucrose and maltose) were the purest obtainable. The results were calculated to correspond to 15 cc. of the original mixture, except where stated otherwise, and are given in terms of mg. of cuprous oxide obtained.

Sherman and Schlesinger[7] compared the amyloclastic and saccharogenic actions of certain amylases, and pointed out clearly the significance of these actions. In this paper, the saccharogenic actions of the enzymes in potato juice, as determined by the amounts of reduced products, are followed and not the amyloclastic (splitting of starch into products which do not give a blue color with iodine). While the two ways of determining the actions undoubtedly overlap to a certain extent, the differences must also be borne in mind. In any event, the chemical changes in either case are not known except with the disaccharides used, so that a strictly quantitative comparison of the actions is not so certain as in other enzyme actions. The possibility of a small amount of cuprous oxide being held in solution by other substances present in the juices must also be considered. Attempts were made to eliminate this and other sources of error by running blanks wherever possible.

The solutions were brought to definite hydrogen ion concentrations before mixing by the addition of sodium hydroxide or hydrochloric acid solutions of various concentrations and tested with the indicators recommended by Clark and Lubs.[8]

### *Experimental Results.*

The properties of the juices from the different lots of potatoes varied somewhat. In general terms, the specific gravities of the juices from new and winter potatoes ranged between 1.025 and 1.035, those from sprouted potatoes (only small, shriveled ones were obtainable) somewhat higher, from 1.035 to 1.040. Almost twice as much juice was obtained from the former as from the latter, approximately 20 to 30 per cent by weight of the original solid with

[7] Sherman, H. C., and Schlesinger, M. D., *J. Am. Chem. Soc.*, 1913, xxxv, 1784.
[8] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1, 109, 191.

the former. The residues after removal of the juice showed moisture contents of between 60 and 70 per cent when dried at 100°. The juice as obtained contained on an average between 3 and 4 mg. of nitrogen per cc. for new potatoes, and somewhat more, 4 to 5 mg., for winter and sprouted potatoes.

The hydrogen ion concentration of the juice as prepared was very close to $10^{-6}$ N or pH 6.0 in every case.[9]

Although the properties of the juice from different lots of potatoes varied somewhat, an attempt was made to make each experiment as complete as possible by running all the necessary blanks. The actions found, therefore, should have general validity. It may also be stated that duplicate determinations agreed satisfactorily.

The results of the actions of the enzymes in the juice upon the constituents of the juice itself and upon added soluble starch solution are given in Table I. The action on the added starch is taken as the difference between the action obtained on the juice plus water and that of the juice plus starch. The results are given as mg. of cuprous oxide obtained by the reducing actions of 15 cc. portions of the solutions. The juice itself was found to have a pH value of 6.0, and the solutions of different acidities were made up by the addition of small quantities of different strengths of acid or alkali. This prevented undue dilution and made the results practically comparable without further calculation. The values of the reductions obtained were corrected for blanks, so that the table shows the actual amount of $Cu_2O$ corresponding to the reducing substances formed during incubation due to the enzyme actions. For the fresh juice the blanks ranged in the extreme cases between 100 and 200 mg. of $Cu_2O$. The enzyme actions did not appear to depend directly on the reducing actions of the juice although it seemed in a number of experiments as if, as with the cabbage, carrot, yellow turnip, and white turnip amylases, the greater the blanks (on the fresh juice) the greater were the actions.

There were considerable variations in the individual experiments, but the large number of determinations made should make it possible to obtain satisfactory mean values. The figures in parentheses in Table I give the mean values for the separate preceding figures.

[9] Cohn, E. J., Gross, J., and Johnson, O. C., *J. Gen. Physiol.*, 1919–20, ii, 145.

TABLE I.

*Saccharogenic Actions of Potato Juice.*

| pH | New potatoes. | | Winter potatoes. | | Sprouted potatoes. | |
|---|---|---|---|---|---|---|
| | Change in juice. | Action on added starch. | Change in juice. | Action on added starch. | Change in juice. | Action on added starch. |
| 2 | 96.6 | 27.5 | | | | |
| | 72.0 | | | | | |
| | (84.3) | (27.5) | | | | |
| 3 | 23.0 | 3.1 | | | | |
| | 59.8 | 7.7 | | | | |
| | 30.7 | | | | | |
| | (37.8) | (5.4) | | | | |
| 4 | 12.4 | 0.6 | 11.5 | 0 | 16.6 | 0 |
| | 23.0 | 6.1 | 6.5 | 0 | | |
| | 10.0 | 0 | 26.9 | 0 | | |
| | 10.5 | 3.9 | 0 | 0 | | |
| | 12.5 | 0.7 | 9.0 | | | |
| | 14.4 | 0 | | | | |
| | 16.1 | 0 | | | | |
| | 18.6 | 0 | | | | |
| | 14.8 | 0 | | | | |
| | (14.7) | (1.3) | (10.8) | (0) | (16.6) | (0) |
| 5 | 3.6 | 10.9 | 46.3 | 0 | 31.6 | 3.3 |
| | 8.7 | 7.3 | | | | |
| | 9.9 | 2.7 | | | | |
| | 16.2 | 1.8 | | | | |
| | 9.0 | 16.4 | | | | |
| | (9.5) | (7.8) | (46.3) | (0) | (31.6) | (3.3) |
| 6 | 36.3 | 20.0 | 37.4 | 26.3 | 44.5 | 39.1 |
| | 16.0 | 27.2 | 49.4 | 28.0 | | |
| | 32.7 | 26.3 | 28.7 | 35.3 | | |
| | 5.7 | 23.8 | 45.5 | 33.0 | | |
| | 6.1 | 31.4 | 44.4 | 32.5 | | |
| | 10.7 | 38.2 | | | | |
| | 14.5 | 11.4 | | | | |
| | 12.5 | 28.8 | | | | |
| | 18.6 | 26.3 | | | | |
| | (17.0) | (25.9) | (41.1) | (31.0) | (44.5) | (39.1) |
| 7 | 18.2 | 15.4 | 30.4 | 17.5 | 23.0 | 39.6 |
| | 20.7 | 36.6 | | | | |
| | 22.8 | 25.1 | | | | |
| | (20.6) | (25.7) | (30.4) | (17.5) | (23.0) | (39.6) |

TABLE I—*Concluded.*

| pH | New potatoes. | | Winter potatoes. | | Sprouted potatoes. | |
|---|---|---|---|---|---|---|
| | Change in juice. | Action on added starch. | Change in juice. | Action on added starch. | Change in juice. | Action on added starch. |
| 8 | 6.4 | 26.0 | 7.0 | 7.7 | 8.1 | 34.6 |
| | 0 | 22.0 | 14.1 | 0.4 | | |
| | 0 | 6.3 | | | | |
| | 0 | 8.0 | | | | |
| | 4.8 | 9.3 | | | | |
| | 3.6 | 14.7 | | | | |
| | 13.1 | 16.1 | | | | |
| | (4.0) | (14.6) | (10.6) | (4.1) | (8.1) | (34.6) |
| 9 | 10.1 | 2.2 | 1.9 | 6.6 | | |
| | 4.4 | 6.3 | | | | |
| | 0 | 3.9 | | | | |
| | (4.8) | (4.1) | (1.9) | (6.6) | | |
| 10 | 0 | 0 | | | | |
| | (0) | (0) | | | | |

Table II shows the ratios of the changes in the juice to the actions on the added starch, with the latter taken as unity, at the different hydrogen ion concentrations.

TABLE II.

*Ratios of Changes in Juice to Actions on Added Starch (as Unity).*

| pH | New potatoes. | Winter potatoes. | Sprouted potatoes. |
|---|---|---|---|
| 2 | 3.1 | | |
| 3 | 7.0 | | |
| 4 | 11.3 | ∞ | ∞ |
| 5 | 1.2 | ∞ | 9.6 |
| 6 | 0.66 | 1.3 | 1.1 |
| 7 | 0.80 | 1.7 | 0.58 |
| 8 | 0.27 | (2.6) | 0.23 |

In addition to the results shown in Tables I and II, some work may be mentioned without entering into detail. In order to determine whether an excess of substrate was present in the juice itself,

a number of samples were hydrolyzed completely with dilute acid. The results showed that for 15 cc. portions in which the blanks (original reducing actions) gave 120 to 150 mg. of $Cu_2O$, reduction after hydrolysis gave 220 to 250 mg. of $Cu_2O$. An excess of substrate capable of forming reducing substances upon hydrolysis was therefore present.

In general terms, the juice from winter potatoes showed greater blanks and somewhat greater actions than the juice from new potatoes, and that from sprouted potatoes greater than that from winter potatoes. These conclusions were confirmed by several experiments with new potatoes which were "aged" for from 1 to 2 weeks in a warm room. The juice showed somewhat greater blanks and greater actions after this "aging" of the potatoes.

Doby[2] had found by his method that sodium fluoride accelerated the action of potato juice on added starch. In attempting to repeat this work, by testing the residue, not merely the supernatant liquid, with iodine, it was found that the action was not accelerated as he stated. Further, by using the reduction method, it was found that the action was retarded by the sodium fluoride at the indicated concentration (0.3 per cent). As pointed out before, the conclusions of Doby were based upon incomplete observations.

### *Discussion of Results.*

The results of Tables I and II may be summarized briefly. In view of the greater number of experiments with new potatoes, the conclusions will be based on the results with these, the other series being used as confirmatory.

With the juice alone, a large action was observed in the acid solution (pH 2), decreasing as the acidity decreased to pH 5, then increasing again, showing a maximum at pH 6 to 7. The actions then decreased with increasing alkalinity, becoming 0 at pH 9 to 10.

The action of the juice on added soluble starch was marked at pH 2, although less than on the juice constituents themselves. This action decreased with decreasing acidity, giving a minimum at about pH 4, increased again with a maximum at pH 6 to 7, and then decreased to 0 at pH 9 to 10.

The two series of actions, on the juice constituents and on the added starch, show definite differences which are also brought out in Table II.

In the first place, it is interesting to note that both sets of actions show maxima at pH 6 to 7, approximately the acidity of the naturally occurring material. This was also observed with the amylase actions of the vegetable juices reported in the previous paper.

In comparing the two sets of actions, at pH 4, the action on the juice constituents is much greater than on the added starch; approximately the same at pH 6 to 7; and reversing at pH 8 where the action on the added starch is much greater than on the juice constituents themselves.

The increase in reducing substances of the juice alone and the juice with added starch is not, in all probability, a simple action. The nature and amounts of the possible products formed were not investigated, so that an accurate quantitative comparison is not feasible. The action on added starch, showing a maximum at pH 6 to 7, may be compared to maxima for the juices of the cabbage, carrot, and white turnip at about pH 6, for yellow turnip, ranging from pH 4 to 7; for pancreatic amylase,[10] pH 7; for malt amylase,[10] pH 4.4 to 4.5; and for *Aspergillus oryzæ* amylase,[10] pH 4.8. This action of potato juice on added starch is without doubt an amyloclastic action. The increase in reducing action of the juice alone may, however, be of more complex nature. The results shown in Tables I and II indicate the possibility that, in addition to the amylase acting upon the starch of the juice, other enzymes may be acting upon other constituents. This view was tested experimentally, and it was found that at least a sucrase and also either sucrose or raffinose or both were present, and that presumably the sucrase hydrolyzed the sucrose or raffinose. No evidence of the presence of a maltase was obtained.

### *Sucrase and Maltase Experiments.*

In order to test for the presence of a sucrase in potato juice, series of experiments similar to those described were set up including additional series containing enough sucrose to make 1 per cent solutions.

[10] Sherman, H. C., Thomas, A. W., and Baldwin, M. E., *J. Am. Chem. Soc.*, 1919, xli, 231.

The mean results of three such series, corrected for blanks, each at pH 4, 6, and 8, are given in Table III.

TABLE III.

*Actions of Potato Juice in Presence and Absence of Sucrose.*

| pH | No sucrose added. | | Sucrose added. | | Difference due to sucrose. | |
|---|---|---|---|---|---|---|
| | Change in juice. | Action on added starch. | Change in juice. | Action on added starch. | Change in juice. | Action on added starch. |
| 4 | 12.2 | 0 | 38.7 | 0 | 26.5 | 0 |
| 6 | 8.1 | 28.0 | 12.0 | 27.5 | 3.9 | 0 |
| 8 | 1.8 | 11.4 | 6.1 | 12.7 | 4.3 | 1.3 |

The results in the last two columns show the presence of a sucrose-hydrolyzing enzyme much more active in the slightly acid (pH 4) solution when acting upon potato juice plus sucrose.

The presence of a maltase in potato juice was tested for in a similar way by using 1 per cent maltose solutions in place of 1 per cent sucrose solutions. The results are given in Table IV.

TABLE IV.

*Actions of Potato Juice in Presence and Absence of Maltose.*

| pH | No maltose added. | | Maltose added. | | Difference due to maltose. | |
|---|---|---|---|---|---|---|
| | Change in juice. | Action on added starch. | Change in juice. | Action on added starch. | Change in juice. | Action on added starch. |
| 4 | 12.2 | 0 | 6.1 | 1.2 | −6.1 | − 1.2 |
| 6 | 12.6 | 27.9 | 14.5 | 10.5 | 1.9 | −17.4 |
| 8 | 1.2 | 9.5 | 1.1 | 3.3 | −0.1 | −6.2 |

No maltase is present in the juice as prepared as far as can be told from these results. The one small positive action obtained on the juice appears to be within the limits of experimental error. The added maltose acts in fact to retard the actions, especially markedly in connection with the action on the added starch. Because of the complexity of the reactions involved in the hydrolysis of starch, this does not appear to be a mass action effect of the products of the reaction retarding further action, but can better be referred to an action between maltose and the amylase, possibly a chemical combi-

nation by which the amylase is prevented from reacting with the starch.[11]

Since the existence of a sucrase was demonstrated in potato juice, it was of interest to determine whether a carbohydrate upon which this enzyme would act was present. As far as known, sucrase acts only upon the carbohydrates, sucrose and raffinose.

The sucrase solution[12] had been prepared from yeast, and the extraneous matter removed by the method of Nelson and Born.[13] This solution exerted no hydrolyzing action on soluble starch or maltose. In order to test the action on potato juice without the interference of the enzymes present in the juice, the latter was heated for 15 minutes in a boiling water bath after the temperature of the juice had reached 80°C. The final temperature was about 90°C. This inactivated the enzymes. The juice was then filtered through paper. It was assumed that the sucrose or raffinose, if present, would not be destroyed and would remain in solution. That some change, presumably partial hydrolysis of some of the starchy materials present, did take place, was shown by the increased reduction (20 to 50 per cent) of the blanks.

In carrying out the tests, the juices as prepared were brought to the requisite hydrogen ion concentrations and 2 cc. of a sucrase solution, made by diluting 1 cc. of the filtered stock solution to 500 cc., were added. 0.5 and 1.0 per cent sucrose solutions similarly treated were run simultaneously. Suitable blanks were also run in every case. After incubation for 20 to 24 hours with toluene as a preservative, the actions were determined by the reduction of Fehling's solution by 5 cc. of the solutions as described above. The results are shown in Table V and are given in terms of mg. of $Cu_2O$ obtained by the reduction by 5 cc. portions, corrected for all blanks.

Each row represents a separate series of experiments. The differences in the sucrose results are due to deterioration of the sucrase solution which stood in a warm room for 3 months. The potato juice results show satisfactory agreement in view of the differences in

[11] Wohl, A., and Glimm, E., *Biochem. Z.*, 1910, xxvii, 349.

[12] A solution of sucrase was supplied by Professor J. M. Nelson of Columbia University for the tests for the presence of sucrose or raffinose.

[13] Nelson, J. M., and Born, S., *J. Am. Chem. Soc.*, 1914, xxxvi, 393.

TABLE V.

*Actions of Yeast Sucrase on Boiled, Filtered, Potato Juice Solutions.*

| | pH | | | | |
|---|---|---|---|---|---|
| | 4 | 5 | 6 | 7 | 8 |
| 0.5 per cent sucrose. | 8.6 | 9.3 | | | |
| 1.0 " " " | (6.6) | 11.5 | 12.2 | 2.2 | 0 |
| Boiled filtered juice. | 14.0 | 15.6 | | | |
| | 12.5 | 9.0 | 9.1 | 4.9 | 1.8 |
| | 12.1 | 10.8 | 9.6 | (8.8) | 1.2 |
| | 8.6 | 11.3 | 7.9 | | |
| | | | 7.8 | 3.9 | 1.2 |

the juices as prepared. The two results in parentheses are probably incorrect.

The results, compared with the results on sucrose, show that the two sets of actions are similar. Optimum actions were obtained over approximately the same range of acidity. This proves that sucrose (or raffinose) is present in the juice, and that its concentration is of the order of magnitude of 1 per cent.[14]

## CONCLUSIONS.

From the actions observed with potato juice alone and in the presence of added starch, it is now possible to state definite conclusions. The marked actions at pH 4 to 5 of the juice alone and also in the presence of the added starch were due to the hydrolysis of sucrose (or raffinose) by the sucrase of the juice. There was very little action upon the starch of the juice itself or upon added starch. At pH 6, the action observed in the juice alone was made up of the action of the sucrase and of the amylase, both on the constituents of the juice; additional action was observed on the added starch. At pH 7 to 8, there was little action of the sucrase, only that of the amylase being observable.

The amylase had an optimum pH of 6 to 7 and acted in the same way upon both the starch occurring in the juice and upon the added

[14] König, J., Chemie der menschlichen Nahrungs- und Genussmittel, Berlin, 4th edition, 1904, 895, 897.

starch. The marked action observed in the more acid solutions was due to the sucrase.

## SUMMARY.

The saccharogenic enzymes present in potato juice were studied. The actions were followed upon the substances present in the juice and upon added sucrose, maltose, and soluble starch.

Sucrase and amylase were found to be present in the juice. No indication of a maltase was obtained.

The sucrase showed optimum conditions for action at pH 4 to 5, the amylase at pH 6 to 7, both upon the starch present in the juice and upon added soluble starch.

The action of a yeast sucrase preparation upon the juice showed the presence of sucrose (or raffinose) in a concentration of the order of magnitude of 1 per cent.

# THE PHOTOCHEMICAL NATURE OF THE PHOTOSENSORY PROCESS.

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## I.

Many animals which are sensitive to light respond by an invariable, characteristic reflex. Typical of such organisms are the ascidian, *Ciona intestinalis*, and the common, North Atlantic, long-neck clam, *Mya arenaria* (Fig. 1). Both of these animals, when illuminated, respond by a vigorous retraction of the siphons. The properties of their sensitivity have been investigated to some extent, and have been described in a series of papers (Hecht, 1918–19, *a*, *b*, *c*, *d*). As a result, an hypothesis has been suggested which accounts for this type of irritability in terms of an underlying, chemical mechanism.

In its essentials this hypothesis involves the behavior of two processes: one, a reversible photochemical reaction; the other, an ordinary, simple, chemical reaction. The light acts on a photosensitive substance $S$ and decomposes it into its two precursors $P$ and $A$. The degree of sensitivity of the sense organ depends, not on the quantity of photosensitive substance $S$, but on the concentration of its precursors $P$ and $A$. Because of this, the amount of fresh precursors necessary for a response is always a constant fraction of the amount of precursors already present in the system. The fresh precursors serve to catalyze the simple, chemical conversion of an inactive substance $L$ into one $T$ which then initiates the nervous impulse. This eventually leads to a contraction of the siphon musculature. The reaction system as a whole may be expressed in the following equations:

$$S \rightleftharpoons P + A; \qquad L \parallel P + A \parallel \rightarrow T$$

in which $\parallel P + A \parallel$ means catalysis by $P$ or $A$, or both.

The evidence which has already been published has established different phases of this hypothesis. (1) Dark adaptation depends on the regular decrease in the concentration of the residual precursors present in the sensory system. This disappearance of precursors is independent of light, proceeds according to the dynamics of a bimolecular reaction (Hecht, 1918–19, *b*), and most probably results in the reformation of photosensitive material. (2) This "dark" reaction has a temperature coefficient of 2.4 for 10°C., similar to those usually found for chemical processes. (3) It is quite characteristic of catalyzed reactions that the velocity of the reaction is a linear function of the concentration of catalyst. Similarly we find that the velocity of the second reaction, $L \rightarrow T$, is a linear function of the concentration of freshly formed precursor catalysts (Hecht, 1918–19, *c*). (4) The simple chemical nature of the catalyzed reaction, $L \rightarrow T$, is evidenced by its quantitative behavior in relation to the temperature. It follows the theoretical expectation according to the Arrhenius equation

$$k_1 = k_0 e^{\frac{\mu}{T}\left(\frac{1}{T_0}-\frac{1}{T_1}\right)}$$

in which the velocity constants ($k$) vary with the absolute temperatures ($T$). In this instance the constant $\mu = 19{,}680$, a value characteristic of simple processes like hydrolyses and saponifications (Hecht, 1918–19, *d*).

In spite of this array of evidence, there is one significant portion of the hypothetical chemical system, the validity of which still remains to be demonstrated. This is the assumption that the reaction $S \rightarrow P + A$ is really photochemical in nature. In other words, it has still to be proved that the action of the light on the sensory process possesses the ordinarily well demonstrated characteristics of photochemical reactions. The present investigation has therefore been concerned with precisely this aspect of the matter; and it is the object of this paper to show that the initial effect of the stimulus is indeed photochemical in nature.

## II.

The experiments were performed with the clam, *Mya arenaria*, which is so common at Woods Hole, Mass. An idea of the appearance of this animal may be obtained from Fig. 1, which is made from

the photograph of a living, medium sized individual expanded in sea water. The extent to which the photosensitive siphon may be protruded is variable; the animal in the figure represents an average condition. On stimulation the siphon is shortened after a clearly defined reaction time. The response is well marked, and involves a movement of the tip of the siphon toward the shell. The amount of this retraction is about 1 cm., often it is more, and it is rarely less than $\frac{1}{2}$ cm. There is never any doubt about the occurrence of a response, nor of the exact moment when the retraction begins. The reaction time may therefore be measured with considerable accuracy.

FIG. 1. From a photograph of a medium sized, living individual of *Mya arenaria*, expanded in sea water. The reproduction is a little less than life size.

The reaction time is not a simple interval. It consists of two distinct periods. The first is the exposure or sensitization period. This is very short, and is the time occupied by the actually necessary exposure to light. The bulk of the reaction time is composed of the second phase, the latent period. During this period it is not necessary for the siphon to be illuminated. Thus an animal which has been exposed to a flash of light of a few hundredths of a second duration will respond in approximately 2 seconds, even though at that moment it is in the dark.

In order to demonstrate graphically this division of the reaction time, I used an optical recording device.[1] The animal is placed vertically in sand in a glass trough filled with sea water. A fine thread connects its siphon tip with a light heart lever as near the axle as possible. To the opposite side of the axle is attached a small mirror which reflects a beam of light into the slit of the recording camera.

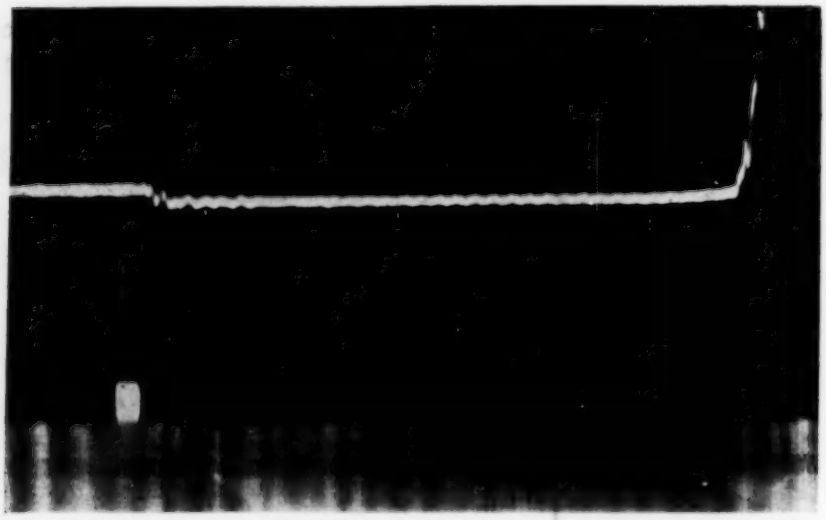

FIG. 2. Optical record of stimulation and response, showing the short exposure period and the long latent period. The white streak running the length of the figure represents the movement of a mirror attached to the siphon tip of *Mya*. The white spot and vertical streak at the left record the duration of the exposure (0.07 second). The siphon retracts nearly 2 seconds after the exposure. The slight irregularity in the siphon record immediately following the exposure is due to an accidental vibration of the delicately balanced mirror caused by the movement of the shutter. The tuning-fork marks 0.02 second.

A contraction of the siphon will be shown by an upward movement of the reflected beam. Passing through a shutter is another beam of light. This beam plays on the expanded siphon, and beyond that, directly on the camera slit. On opening the shutter, the beam will

[1] Dr. D. J. Edwards was kind enough to let me use his optical recording apparatus, and to give me much of his time and help in the making of records, for all of which I wish to express my gratitude.

therefore stimulate the animal, and at the same time record the exact duration of the exposure on the moving film. The shadow of a vibrating tuning-fork is also focused on the slit, so as to furnish a time record.

One of the records obtained in this manner is given in Fig. 2. With an exposure of 0.07 second at this intensity, the retraction of the siphon appears only after a latent period of nearly 2 seconds. A record like that of Fig. 2 is striking testimony of the composition of the reaction time. Since it is our purpose to study the photochemical aspect of photic sensitivity, our attention must therefore be devoted to the relatively short exposure period during which the reception of the light takes place.

## III.

Before taking up the nature or the details of the experiments, it is necessary to describe the apparatus which is used for the accurate control of short exposures. In principle the mechanism is that of a focal plane shutter. Its construction may be described with the help of the three views in Fig. 3. Essentially the shutter consists of a piece of black cardboard C, having a variable aperture A which moves with a definite speed past an opening O, through which a beam of light is directed. The duration of the exposure depends on the velocity of the cardboard and the size of the aperture. The rest of the apparatus serves merely to control these two factors accurately and easily.[2]

The variable aperture is obtained by using slides S, each having a different sized opening—all slides, however, having the same weight. A slide is placed in the apparatus by slipping it into the raised grooves G; it is kept in place by them with the help of the raised end-piece P. The front of the shutter may be easily removed for the exchange of slides by turning the thumb screws T. The cardboard C which holds the slide is attached at the bottom to a cylindrical wooden rod R. As it moves across the field the cardboard slides in a simple groove at the top of the shutter, and the wooden rod slides in a metal tube Z

[2] I made this apparatus by converting a focal plane shutter originally constructed by Mr. J. G. Hubbard. I take this opportunity of thanking Mr. Hubbard for much in the way of advice and material assistance in the building of apparatus used in this work.

at the bottom. When it is at the left of the shutter, the rod is held by a small projecting spring clip F, which may be released by hand, or more conveniently by an air bulb. To the ends of the cylindrical

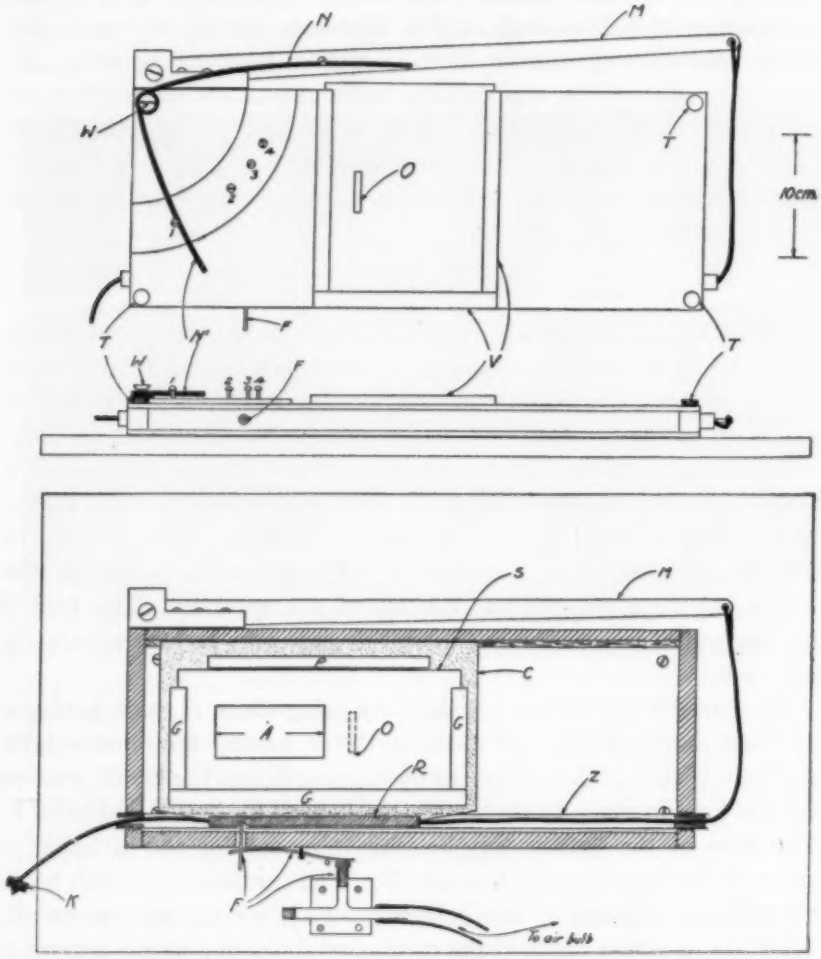


FIG. 3. Three views of the shutter. The upper drawing is the front view; below that is the bottom view; and below that is a partial section through the shutter. The letters are explained in the text. In the upper drawing the details of the spring release clip F are omitted, because they are given in the lowermost view. Similarly the board to which the shutter is attached is also omitted from the upper drawing.

rod R is attached strong twine, which on the left connects with a knob K, and to the right leads to the projecting bar M. Therefore an upward movement of the bar results in the sliding of the cardboard across the length of the shutter.

The motion of the projecting bar is due to the release of the heavy brass wire spring NN′ wound several times around the screw W. The tension of the spring and therefore the speed of the shutter may be varied by placing the arm N′ of the spring against the appropriate raised screw head 1, 2, 3, or 4. The calibration of this movement is accomplished by recording the excursion of the tip of the bar M on a rapidly moving kymograph. Several such records gave identical results. A few hundredths of a second after being released, the shutter slide moves with a constant velocity, which in these experiments is 67.5 cm. per second. The exposure may therefore be computed from the width of the aperture in the slide, by multiplying the width by the fraction of a second during which the slide moves 1 cm. (= 0.0148 second). The front and rear boards of the shutter have raised grooves V for the insertion of cards having different sized stationary openings O. In this manner, the dimensions of the beam of light may be varied. In these experiments the size of the stationary opening is $5 \times 35$ mm.

To get the shutter ready for use, the opening O is temporarily covered with a black card, and the knob K is pulled until the shutter slide C is brought to the left of the shutter. Here it is caught by the release clip F and held in position. The movement of the shutter slide C of course pulls the bar M down to the top of the shutter against the tension of the spring NN′. The temporary card is then removed, and the shutter is ready for an exposure. At the proper moment the spring clip is released, and at once the spring NN′ exerts its action. The bar M is jerked rapidly upward and the shutter slide C is shot past the opening O, thus effecting the proper exposure.

When set up, the shutter as a whole is placed in the front wall of a light-tight compartment Y (Fig. 4), containing a 250 watt concentrated-filament Mazda lamp. The center of the beam through the opening O is a few millimeters above the level of a long table B. This is so that the beam will be exactly centered on the siphon of an animal in a dish of sea water standing on the table. The general arrangement

of the apparatus is shown in Fig. 4. The top of the table B is painted black except for a white streak parallel to the long axis of the light beam. The white streak is a background on which to view the movement of the siphon. In addition the streak is graduated in centimeters marking distances from the center of the source of illumination. In this way the siphon of the animal may be placed at exactly the desired distance from the light, where it will receive the proper exposure from the shutter.

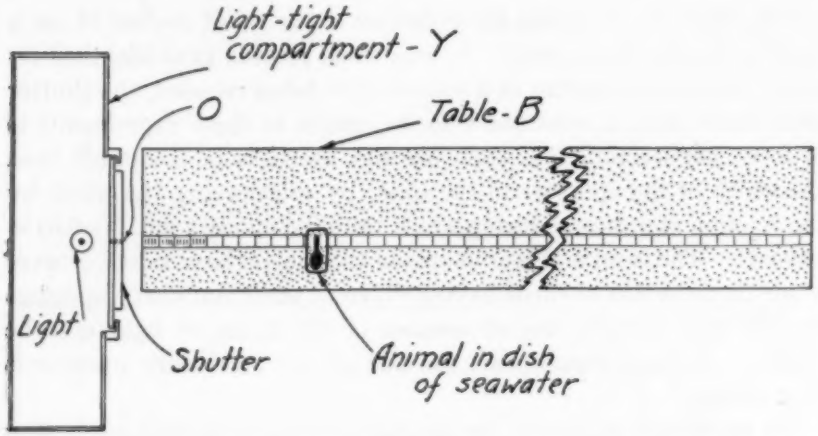


FIG. 4. Arrangement of apparatus in the dark room. The air bulb attached to the spring release clip of the shutter by means of rubber and glass tubings is not shown in the figure. With the help of this bulb and tubing, an exposure can be effected from any position at the work table.

IV.

The first experiments with which I undertook to test the photochemical nature of the light sensitivity were concerned with the relation between the exposure time and the minimum intensity necessary to produce a response. Since the discovery of the Reciprocity Law by Bunsen and Roscoe in 1862, its validity has been demonstrated for a variety of purely photochemical reactions. It obtains whenever the quantity of photic energy necessary to produce a given chemical effect is constant, whether the intensity is low and the exposure long, or the reverse. There are complex reactions, partly photochemical

in nature, which do not obey the reciprocity rule (Schwarzschild, 1899). However, when a process does proceed according to this dictum, it is highly probable that its basis is a simple photochemical reaction.

The usual procedure in investigations of this kind is to vary the intensity and to determine the time required to produce a given effect. This course was adopted with *Ciona*, and the results bore out the Bunsen-Roscoe expectation (Hecht, 1918–19, *a*). Such a technique is entirely out of the question with *Mya*. The exposure required is so short that the errors of measurement would be too great. It is simpler to keep the exposure time under control, and measure the minimum intensity necessary to elicit a response with different exposures. Six exposures were chosen for experimentation. Their durations are given in Table I, first column.

TABLE I.

*Relation between Exposure Duration and Minimum Intensity Necessary to Elicit Response.*

| Exposure ($t$). | Intensity ($I$). | $I \cdot t$ |
|---|---|---|
| *sec.* | *meter candles* | *meter candle sec.* |
| 0.016 | 334 | 5.34 |
| 0.023 | 238 | 5.47 |
| 0.030 | 194 | 5.82 |
| 0.053 | 112 | 5.94 |
| 0.073 | 76 | 5.55 |
| 0.104 | 54 | 5.62 |
| Average | | 5.62 |

Eight animals which had been thoroughly dark-adapted were used. Taking a given exposure, I subjected each animal in turn to it, and noted whether it responded or not. After each animal had been given a rest of at least 15 minutes, it was again exposed, nearer or farther from the light depending on the individual's previous response. In this way the distance at which a response was elicited was gradually approximated to the distance at which no response could be elicited. I considered a determination as finished when the "no response" distance was 1 cm. farther than the "response" distance. Frequently

the last readings were repeated a few times. An example of an experiment which will indicate the procedure is given in Table II. The entire series of experiments took 6 days, the same animals being used throughout. They were in good condition after the experiments, and remained alive in the laboratory for many days afterwards.

From the determination of the individual minimum distance, it is simple to calculate the minimum intensity by the inverse square law. This was done for each animal for every exposure before an average

TABLE II.

*Determination of Minimum Stimulating Distance.*

Animal 120, July 18, 1919, exposure, 0.053 second.

| Time. | Distance. | Response. |
|---|---|---|
| | cm. | |
| 10.07 | 90 | R. |
| 10.34 | 100 | R. |
| 10.52 | 100 | R. |
| 11.14 | 120 | R. |
| 11.41 | 140 | R. |
| 11.57 | 150 | R. |
| 12.18 | 150 | R. |
| 12.34 | 160 | N. R. |
| 2.22 | 155 | N. R. |
| 2.46 | 152 | R. |
| 3.00 | 153 | R. |
| 3.16 | 154 | N. R. |

R. = Response elicited.
N. R. = No response elicited.

value was made. A summary of the experiments, giving the average figures, is shown in Table I and graphically in Fig. 5. The third column of Table I shows clearly enough that the Reciprocity Law of Bunsen and Roscoe holds true for this form of photic sensitivity. The curve drawn in Fig. 5 is a theoretical one on the assumption that the photochemical effect ($E$) is a function of the intensity ($I$) and the exposure ($t$), so that

$$Ek = I \cdot t = 5.62$$

$k$ being a constant. The deviations of the average experimental points from the theoretical hyperbola of Fig. 5 are not great. In fact the probable error of any *individual* determination is 6.7 per cent of the mean value assumed in drawing the hyperbola in Fig. 5.

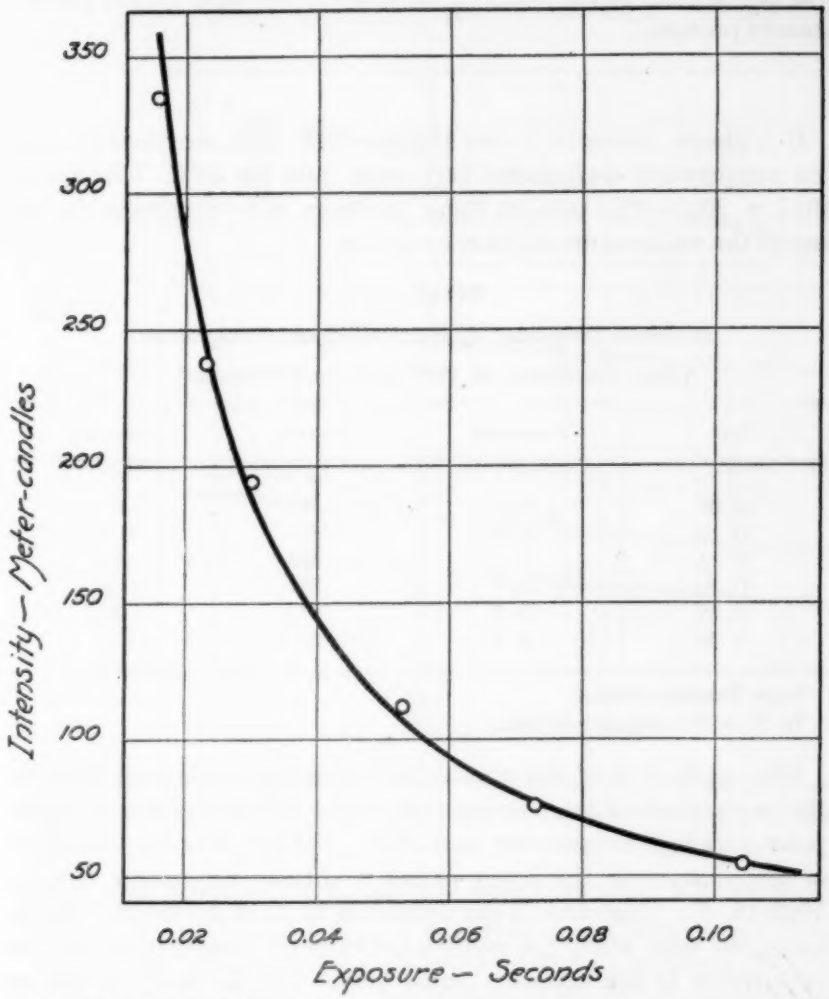


FIG. 5. Relation between exposure and minimum intensity necessary for a response. The points are the average experimental values. The curve is an hyperbola, $I \cdot t = 5.62$, drawn from the theoretical expectation of the Bunsen-Roscoe law.

On the basis of these results, there seems good reason to believe that the initial action of the light on the sense organ is photochemical in nature. This is precisely what we have assumed in our hypothesis. However, there is still another test possible in this connection, and it was also applied in the study of the effect of the light on the photosensory process.

V.

It is almost axiomatic to say that photochemical reactions possess low temperature coefficients, very near 1.00 for 10°C. (Sheppard, 1914, p. 304). The obvious thing, therefore, is to determine the effect of the temperature on photoreception.

TABLE III.

*Minimum Stimulating Distance at Different Temperatures.*

Animal 186, August 20, 1919, exposure, 0.016 second.

| Time. | Temperature. | Distance. | Response. |
|---|---|---|---|
| | °C. | cm. | |
| 11.05 | 24.3 | 57 | R. |
| 11.10 | 24.5 | 58 | N.R. |
| 11.15 | 24.3 | 57 | R. |
| 11.20 | 24.9 | 58 | N.R. |
| 11.25 | 24.5 | 57 | R. |
| 11.30 | 24.1 | 58 | N.R. |

R. = Response elicited.
N. R. = No response elicited.

Here again it is of the utmost importance to distinguish between the two periods of the reaction time. The reaction time as a whole possesses a high temperature coefficient. For this, however, the effect of temperature on the latent period is entirely responsible (Hecht, 1918–19, *d*). Therefore, if our results are to be of the desired significance, we must study the relation between the temperature and the occurrences in the exposure period only. For the same reasons as before, this resolves itself into a determination of the minimum intensity necessary to elicit a response at different temperatures with a given exposure.

The experiments were conducted as previously outlined, except that 5 minutes were allowed between tests. This time has been found sufficient for the complete recovery from a single stimulus. Using a constant exposure of 0.016 second, I determined the minimum intensity necessary to elicit a response at four different temperatures. When the minimum stimulating distance was found, the determina-

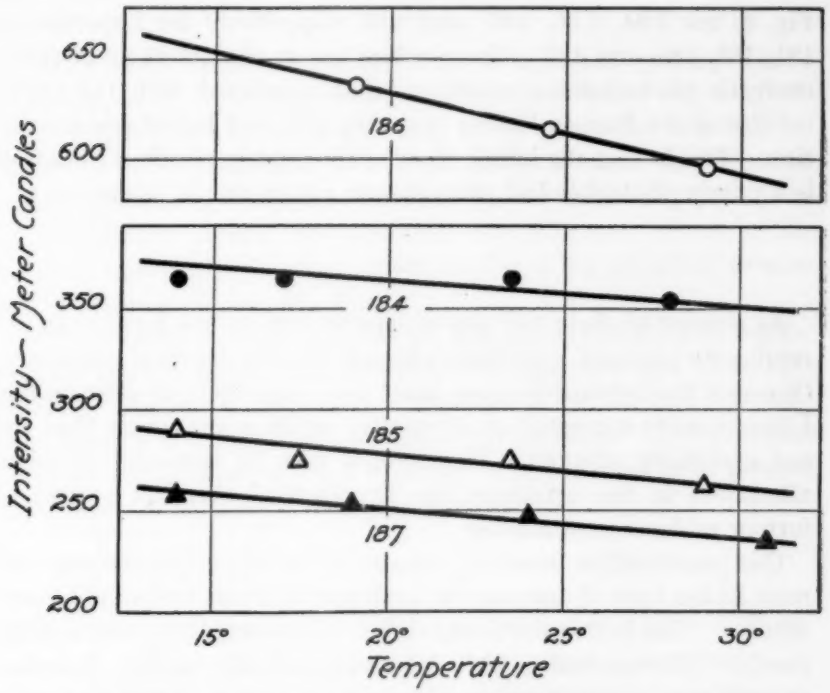


FIG. 6. Relation between the temperature and the minimum intensity necessary for stimulation. The points are single determinations for each experiment shown.

tion was checked at least twice. Table III gives the details of a portion of one such experiment, wholly typical of the others. Proper precautions were, of course, observed for the maintenance of a fairly constant temperature, etc. In this respect *Mya* is a particularly good experimental animal, because it helps to stir the sea water by means of its own continuous water current.

After some preliminary tests, four carefully controlled experiments were made, several days apart. The results were so uniform that further experimentation was deemed unnecessary. The data for the individual experiments are given graphically in Fig. 6. It will be seen that the effect of the temperature of the animal on the minimum stimulating intensity is practically negligible. The temperature coefficients for 10°C. (15–25°), calculated in the ordinary way from Fig. 6, are 1.04, 1.06, 1.07, and 1.06 respectively for Experiments 184, 185, 186, and 187. These values are so characteristic of endoenergetic photochemical reactions, that, combined with the applicability of the Bunsen-Roscoe law, they can lead to but one conclusion. This is that the initial effect of the light in photic stimulation is a purely photochemical phenomenon rather simple in nature.

## VI.

As a result of these two sets of experiments we are justified in accepting the proposed hypothesis with a reasonable degree of confidence. Of course the hypothesis is not final. At each step in its construction I have usually suggested an alternative which is less simple than the one eventually adopted. Therefore, it may be necessary to make alterations in the details of the hypothetical chemical system as further evidence accumulates.

One consideration, however, remains of paramount significance and must be the basis of any possible explanation of this kind of photosensitivity. This is that the mechanism of photoreception is not a single process. Corresponding with the division of the reaction time into an exposure or sensitization period and a latent period, there is a fundamental division of the underlying machinery into an initial photochemical reaction and a consequent ordinary chemical reaction. This duality is patent in every experiment with *Ciona* and *Mya*.

Whether the initial photochemical reaction is strictly reversible or only pseudoreversible depends in a large measure on the relation between the primary and secondary reactions of photoreception. It is often as compatible with the data to assume the primary reaction to be

$$S \xrightarrow{\text{light}} P + A$$
$$S \xleftarrow{\text{"dark"}} P + A'$$

in which $A'$ is an accessory precursor different from $A$, as it is to assume it to be

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A.$$

If, as we have postulated, the secondary is catalyzed by the products of photolysis of the primary reaction, then the primary reaction is to be considered as strictly reversible. If, however, the reaction between the two is conceived to be in the nature of a catenary chemical interaction, then a pseudoreversible reaction is a more likely possibility for the primary process than a strictly reversible one. Even in this instance, a photochemical reaction is feasible, if the secondary process itself is assumed to be reversible.

None of these alternatives, however, appear very attractive because of their innate complexity. This becomes especially true when it is necessary to make a mathematical analysis of data in order to compare theoretical expectation and actual performance. The proposed hypothesis of photoreception is therefore to be preferred in its present form, unless some glaring discrepancy arises.

## VII.

Aside from their significance with regard to an hypothesis of photoreception, these experiments involve some general conceptions of rather wide application. In the study of the responses of organisms much agitation has resulted over the difference between those individuals which are sensitive to the continuous action of light, and those which are sensitive to a rapid change in the intensity of the light only. The distinction as it is usually made rests on the assumption that for the one group the effective stimulus is a definite quantity of light, whereas for the other group the effective stimulus is the *rate of change* of the intensity. The conception underlying this differentiation, however, is as fallacious as it has been common. I have no desire to enter into a controversy which has been continued much too long. But the

experiments on *Ciona* (Hecht, 1918–19, *a*) and *Mya* have demonstrated that the distinction on which this controversy rests is without any but the most superficial basis.

The facts are simple enough. Blaauw (1909) and Fröschel (1909) demonstrated that the orientation of certain plants obeys the Reciprocity Law of Bunsen and Roscoe; therefore a definite amount of light is required for a stimulus. Loeb (1918) and his associates proved the same to be true for the animals with which they experimented. All these organisms are, of course, to be classed in the group which responds to an obviously continuous source of illumination. On the other hand, *Mya* and *Ciona* belong decidedly in the group of organisms which is sensitive to light only when the illumination intensity has been increased rapidly. And yet both *Mya* and *Ciona* must receive a definite amount of light before they respond. In *Ciona* this is approximately 5,000 meter candle seconds; and in *Mya*, as we found in this paper, the required energy is about 5 meter candle seconds. The stimulus for both groups of organisms is therefore the same, a definite quantity of light energy producing a specific photochemical effect. Certainly no distinction can be made between them on this score.

One point remains to be elucidated. Since both groups of organisms require fundamentally the same stimulus, how is it that animals like *Mya* and *Ciona* respond apparently to sudden illumination only? This is indeed a paradoxical situation, yet the answer to the question is simple. The explanation depends on the reversible character of the sensory process.

The presence of a regenerative mechanism in the sense organ is shown by the course of dark adaptation. The photosensitive material decomposed by the light is automatically regenerated as soon as some products of decomposition accumulate. The velocity of this regenerating reaction depends on the concentration of the precursor decomposition products. Therefore a little time must elapse before the effect of the regenerating reaction will become apparent. It is precisely during this short interval of time that the required amount of energy must be received by the sense organs in order to produce a response.

Physically speaking, this energy requirement means that a definite mass of sensitive substance is decomposed by the light. When the energy is delivered rapidly, that is when a high illumination is attained suddenly, the necessary photosensitive substance is decomposed before the regeneration reaction sets in. But if the energy is delivered slowly, sufficient sensitive substance to produce a response cannot be decomposed before the "dark," regeneration reaction becomes effective. The "dark" reaction, once under way, proceeds slowly or rapidly depending on the rate at which the light decomposes the sensitive substance into its precursors. As a result, a stationary state is attained analogous to a condition of true equilibrium, in which no amount of exposure to light can form enough precursors to initiate a response. Even if the intensity continues to increase to a very high value, no effect can be produced as long as the increase is slow, simply because a new stationary state of the opposing reactions will be reached if enough time is allowed.

The time interval during which the necessary amount of energy must be delivered is known experimentally. In *Ciona* the 5,000 units of energy must be received within about 10 seconds; in *Mya* the required five units must enter the sense organ in less than 1 second. The time limit is thus greater in *Ciona* than in *Mya*. This time interval, as we have said, depends on the speed with which the regeneration reaction proceeds. Therefore the regeneration reaction in *Ciona* should be slower than in *Mya*. This is indeed the situation. Dark adaptation is wholly dependent on the regeneration reaction (Hecht, 1918–19, *a*, *b*). In *Ciona*, dark adaptation requires about 4 hours, whereas in *Mya* the same process is complete in about 35 minutes. The time limit for the energy delivery is thus proportional to the velocity of the "dark" reaction which regenerates the sensitive substance.

The conclusion is clear. In spite of the apparent sensitivity of animals like *Mya* and *Ciona* to sudden illumination the effective agent for this sensitivity is a definite quantity of energy which obeys the Reciprocity Law of photochemistry. The fact that this amount of light energy must be received by the sense organs in a limited time is merely a concomitant of the additional circumstance that the photochemical reaction is reversible. The *rate of change* of the light inten-

sity is decidedly not the effective stimulus. Therefore the controversial division of organisms into the two groups previously mentioned, though traditionally sacred, is fundamentally without significance. The effective stimulus in photic irritability is of the same nature whether the response is to a light which is obviously continuous, or to a light whose intensity is suddenly augmented.

## SUMMARY.

1. In order to produce a response in *Mya*, the minimum amount of light energy required is 5.62 meter candle seconds. This energy follows the Bunsen-Roscoe law for the relation between intensity and time of exposure.

2. The necessary minimum amount of energy varies but little with the temperature; the temperature coefficient for 10°C. is 1.06.

3. In view of these facts it is concluded that the initial action of the light is photochemical in nature. This substantiates the hypothesis previously suggested to account for the mechanism of photoreception.

4. The constant energy requirement for stimulation of *Mya* shows that the traditional division of animals into those which respond to a constant source of light and those which respond to a rapidly augmented light is without any fundamental significance for sensory physiology.

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# THE FREE ENERGY OF BIOLOGICAL PROCESSES.*

## Preliminary Paper.

By GEORGE A. LINHART.

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(Received for publication, October 5, 1919.)

Any process in nature is accompanied by a transfer of energy. Part of this energy can be used for doing useful work; part of it is dissipated or rendered latent. Moreover, in the burning of a substance, such as coal, only a small percentage of the available energy is at present utilized; the bulk of it is lost in the form of heat. This immense loss of energy is no doubt due to the clumsiness of our technique and to the imperfection of the work engines now in common use. This leads us to inquire whether similar processes in living organisms are more efficient. An investigation has therefore been undertaken to find the ratio of the energy supplied and the maximum work obtainable in life processes. As a suitable subject for this purpose, it was decided to study the fixation of nitrogen through the agency of microorganisms, such as *Azotobacter*.

Every biological process presents three problems for solution: first, the transfer of energy, which depends solely on the initial and final states of the system; second, the mechanism of the reactions involved; and, third, the rates of these reactions.

Up to the present time, biologists and agriculturists have been interested chiefly in the mechanisms and the rates of life processes. However, from this it must not be inferred that they have underestimated the importance of the study of energy relations in life processes. Such a study requires a thorough knowledge of general chemistry (organic and inorganic), and of thermodynamics and the application of its principles to chemical processes. In this day of

* This investigation is being conducted under the direction of Dr. Charles B. Lipman and with the cooperation of Dr. A. R. Davis of this laboratory.

specialization, it would be too much to expect biologists to possess this knowledge in addition to the mastery of their own special field. They are, however, willing to cooperate with physical chemists to bring about the desired progress in biological science. Unfortunately, whenever they present their problems to the physical chemist, they are always met with the statement: "Biological processes are so highly complex that they are not susceptible to thermodynamic treatment."

Biologists and agriculturists cannot, of course, get satisfactory information on this subject from text-books on physical chemistry, since these embody principally the views and guesses of physical chemists of past generations and are interesting from an historical standpoint only. In fact, it was only within recent years that the first serious attempt was made to base even chemical calculations systematically on thermodynamic principles. This was done by Lewis,[1] and Lewis and Gibson.[2] It is hoped that by the use of their methods we may be able to do for general physiology, in a measure at least, what Lewis has done for general chemistry.

In order to find the maximum or free energy of any chemical process, such as the transformation of mannite into carbon dioxide and water, which certain bacteria utilize for the work required in decomposing sulfates, phosphates, nitrates, or in the "fixing" of nitrogen, we must know not only the change in the heat content attending the process, but also the change in entropy from the absolute zero up to the given temperature, $T$, at which the process takes place. We can then calculate the free energy of the process with the aid of the thermodynamic equation

$$\Delta F = \Delta H - T\Delta S \qquad (1)$$

where $\Delta F$ denotes the free energy charged, $\Delta H$ the change in heat content, and $\Delta S$ the change in entropy of the isothermal process in question.

The thermochemical data found in the literature, although very extensive, are, with few exceptions, extremely inaccurate, while the measurements of usable entropies have only recently been started

[1] Lewis, G. N., *J. Am. Chem. Soc.*, 1913, xxxv, 1.

[2] Lewis, G. N., and Gibson, G. E., *J. Am. Chem. Soc.*, 1917, xxxix, 2554.

under the direction of Lewis.[3] However, the compounds in whose entropy Lewis and his coworkers are at present interested are not of the type which suit our purpose. Furthermore, it may take many years before the data which we desire become available. We have, therefore, decided to set up a specific heat apparatus of our own, as well as a high precision bomb calorimeter outfit, for the accurate measurements of heats of combustion of carbohydrates and other organic compounds directly connected with our work. In the meantime, we are conducting experiments with nitogen fixation bacteria to determine the most favorable conditions for the obtaining of interpretable data.

It may be of interest to show just how the free energy calculations of a biological process are made from approximate data found in the literature.

If we wish to find the efficiency of the process of the fixation of nitrogen by *Azotobacter* in a 0.1 molal solution of mannite, it is only necessary to consider the mannite in the thermodynamic calculations, since the concentration of the essential salts added is extremely small compared with that of the mannite itself.

(*a*) *Calculation of* $\Delta H$ *for the Reaction.*—

$$6\,C + 14\,H + 6\,O = C_6H_{14}O_6$$

This is equivalent to the heats of formation of 6 mols of $CO_2$ plus 7 mols of $H_2O$ minus that of the reaction

$$C_6H_{14}O_6 + 6\tfrac{1}{2}\,O_2 = 6\,CO_2 + 7\,H_2O$$

or

$$\Delta H = 6(-94{,}250) + 7(-68{,}280) - (-728{,}400) = -315{,}060 \text{ calories}$$

(*b*) *Calculation of* $T\Delta S$ *for the Same Reaction.*—In order to calculate the entropies of the substances involved in the above reaction, we must know their specific heats from the absolute zero up to 25°C., the temperature at which the reactions take place. The entropy of each substance is related to the average atomic heat at constant volume, $C_v$, by the thermodynamic equation:

$$S = \int_0^T C_v d\ln T = 2.3 \int_0^T C_v d \log T \qquad (2)$$

[3] The measurements are being made in the chemical laboratory of the University of California under the direction of Dr. G. N. Lewis.

Although we have sufficient data from which to calculate the entropies of $CO_2$ and of $H_2O$, there are no such data available for mannite. We can, however, obtain an approximate value for our present purpose by comparing its average atomic heat at 25°C. (the only value found in the literature) with those of other substances whose entropies are known and whose average atomic heats at 25°C. are approximately of the same magnitude as that of the mannite. For this purpose, we may plot $C_v$ against log $T$ for solid crystalline $C_6H_6$ and for graphite. Since for a certain class of substances these curves do not intersect, but approach the absolute zero in the same general way, we may locate between these two curves the average atomic heat curve for mannite.[4]

The area under this curve amounts to approximately 0.95. This multiplied by the number of atoms and by 2.3 (see Equation 2) gives 56.8 entropy units. Subtracting from this value the entropies of 14 hydrogen atoms (222.6), 6 oxygen atoms (144.6), and 6 carbon atoms (7.8), we obtain −318 entropy units as the value of $\Delta S$ for mannite. We then have for the free energy of formation of 1 mol of solid crystalline mannite from its elements at 25°C.

$$\Delta F = \Delta H - T\Delta S = -315{,}060 - (-94{,}760) = -220{,}300 \text{ calories}$$

Finally to obtain the free energy of 1 mol of mannite when dissolved in 10 kilos of water, we may assume, in the case of substances like mannite, that the activities are proportional to the concentrations, so that for the free energy of dilution from saturation to 0.1 molal

$$\Delta F' = -RT\ln\frac{1.49}{0.1} = -1{,}600 \text{ calories}$$

Adding this to the above value, we obtain −221,900 calories. With this value we can now calculate the available or free energy of the reaction.

$$C_6H_{14}O_6 + 6\tfrac{1}{2}\,O_2 = 7\,H_2O + 6\,CO_2$$

or

$$\Delta F' = 7(-56{,}620) + 6(-94{,}360) - (-221{,}900) = -740{,}600 \text{ calories}$$

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[4] The average atomic heat for mannite is obtained by multiplying its specific heat, which is given in the literature for 25°C., by the molecular weight and dividing the product by the number of atoms in the molecule.

This would be the maximum amount of energy at the disposal of the *Azotobacter* in 10,182.1 gm. of solution, 182.1 gm. of which is mannite, if the latter were all converted into $CO_2$. To be sure, a certain amount of the carbon of the mannite is utilized by the bacteria in the building of their proteins; the exact amount so combined will need to be determined experimentally. For the present, let us assume that all the carbon in the mannite is converted into $CO_2$.

As previously noted in the introduction, a thermodynamical calculation of transfer of energy involves a knowledge of the initial and final states of the system. In the present instance, we have mannite as the initial state—the final state is not yet known. For the purpose of this calculation, we may assume the final product to be hydrated ammonia. Under this assumption, $-740{,}600$ calories would be sufficient energy to form about 10 mols of ammonia from water and nitrogen gas; that is, if the bacteria acted directly or indirectly merely as catalytic agents. As a matter of fact, it is shown in Greaves'[5] summary of nitrogen fixation by *Azotobacter* that the amount of nitrogen fixed is only about 1 per cent of the weight of mannite, or, in molal proportions, for every mol of mannite used up only about 0.1 mol of ammonia is formed. According to the source above quoted, we would obtain about 0.1 mol of ammonia instead of 10 mols, or only about 1 per cent of the total available energy is utilized in the fixation of nitrogen.

[5] Greaves, J. E., *Soil Science*, 1918, vi, 181.

# APPARATUS FOR MEASUREMENT OF OXIDASE AND CATALASE ACTIVITY.

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(Received for publication, November 25, 1919.)

On account of some undesirable features of the simplified Bunzel oxidase apparatus,[1] the author has designed a simple apparatus (Fig. 1) incorporating the advantages of both the simplified Bunzel apparatus and the original design.[2]

The simplified Bunzel oxidase apparatus makes no provision for the absorption of $CO_2$ other than by the reagents used. In some reactions the $CO_2$ production cannot be disregarded without error. Bunzel gives data on this point.[2]

This source of error is removed in the new design by using a caustic tube and alkali to absorb the $CO_2$ produced during the reaction. 1 cc. of 0.1 N alkali is placed in the caustic tube, and on shaking the apparatus the liquid surges back and forth beneath the tube, forcing the air over the alkali.

The apparatus has a volume of 68 cc. measured to the zero of the middle graduated tube. The volume of glass comprising the boat is 3 cc., to which is added 1 cc. of alkali, 2 cc. of plant juice or dilution, and 5 cc. of reagent and buffer solution, totaling 11 cc. In case it is desired to use the apparatus without the alkali tube, the total volume of liquid should be 11 cc. This allows 57 cc. of air space in which a difference of 1 cm. between the mercury levels in the manometer corresponds to a change of 0.75 cc. in volume at 760 mm. pressure. The manometer is graduated for both positive and negative pressures.

The apparatus can be conveniently used for the determination of catalase activity.

[1] Bunzel, H. H., *J. Biol. Chem.*, 1914, xvii, 409.

[2] Bunzel, H. H., *J. Am. Chem. Soc.* 1912, xxxiv, 303.

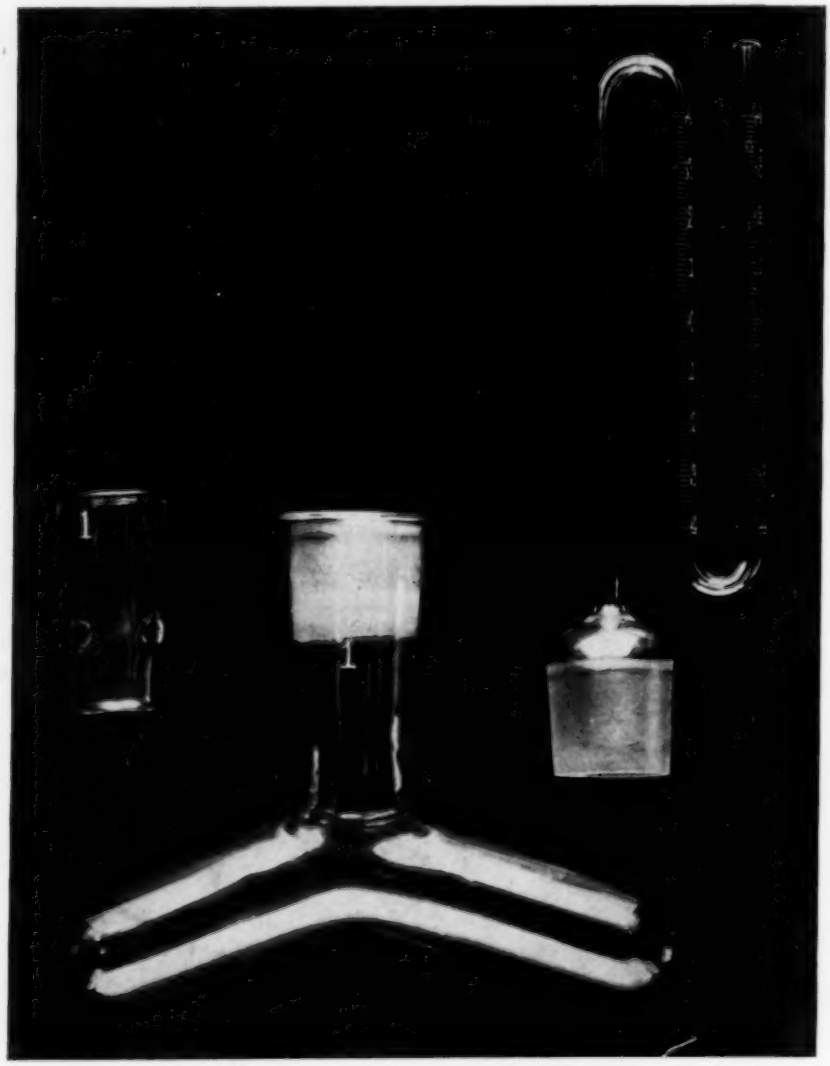

FIG. 1. Simplified apparatus for measurement of oxidase and catalase activity, with tube for $CO_2$ absorption.

# INFLUENCE OF A SLIGHT MODIFICATION OF THE COLLODION MEMBRANE ON THE SIGN OF THE ELECTRIFICATION OF WATER.

By JACQUES LOEB.

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I.

When we separate a solution of an electrolyte from pure water by a collodion bag the water will as a rule diffuse into the solution. In a preceding paper[1] it was shown that the forces determining this diffusion are different for concentrations of electrolytes below or above a certain value (which is for certain sodium salts about M/16). The forces causing the diffusion of water into the solution below this critical concentration are predominantly electrical, while the forces causing the diffusion above the critical concentration are predominantly (or perhaps exclusively) molecular.

The electrical forces of diffusion depend upon the sign, the valency, a third property of the ions (which we arbitrarily designated as their radius), and in addition upon the concentration of the ions in solution. There is still another variable to be considered; namely, the nature of the membrane. We have already called attention to the fact that a collodion membrane which has once been treated with a gelatin solution shows a different osmotic behavior from a membrane not treated with gelatin. The gelatin treatment consisted in this that the collodion bags with which the experiments were made were filled over night with a 1 per cent gelatin solution (isoelectric or nearly isoelectric). The next day the gelatin solution was poured off and the interior of the bags washed out about six times or more with warm water to remove as much gelatin as possible. They were then put for days into water to dissolve still more and remove the last traces of gelatin.

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173.

Such bags seemed to retain the effect of the gelatin treatment for a long time, and continued use and subsequent washing did not seem to remove this after effect of the gelatin treatment.

When collodion bags which had received this gelatin treatment were filled with solutions of electrolytes (of the theoretical osmotic pressure of that of a M/64 sugar solution) and were dipped into beakers with distilled water, the influence of the nature of the electrolyte upon the initial rate of diffusion of water into the bag could be expressed by the following two rules.[2]

1. Solutions of neutral salts possessing a univalent or bivalent cation influence the rate of diffusion of water through a collodion membrane, as if the water particles were charged positively and were attracted by the anion and repelled by the cation of the electrolyte; the attractive and repulsive action increasing with the number of charges of the ion. The same rule applies to solutions of alkalies.

2. Solutions of neutral or acid salts possessing a trivalent or tetravalent cation influence the rate of diffusion of water through a collodion membrane as if the particles of water were charged negatively and were attracted by the cation and repelled by the anion of the electrolyte; the attractive and repulsive action increasing with the number of charges of the ion. Solutions of acids obey the same rule.

When the same experiments were repeated with collodion bags which had not come in contact with gelatin, the influence of the electrolytes mentioned in Rule 1 on the diffusion of water was the same as when the membrane had been treated with gelatin. Rule 2, however, was not valid when the collodion membranes had *not* been treated with gelatin. It was of interest to discover the cause of this difference.

The curves in Figs. 1 and 2 show that Rule 1 holds also for collodion membranes *not* treated with gelatin. The solutions of salts used were neutral or slightly alkaline (in the case of $Na_3$ citrate). The abscissæ in the figures are the logarithms of the concentration, the ordinates the height to which the level of liquid in the manometer rose in the first 20 minutes. The curves in Fig. 1 show that the levels

[2] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 717.

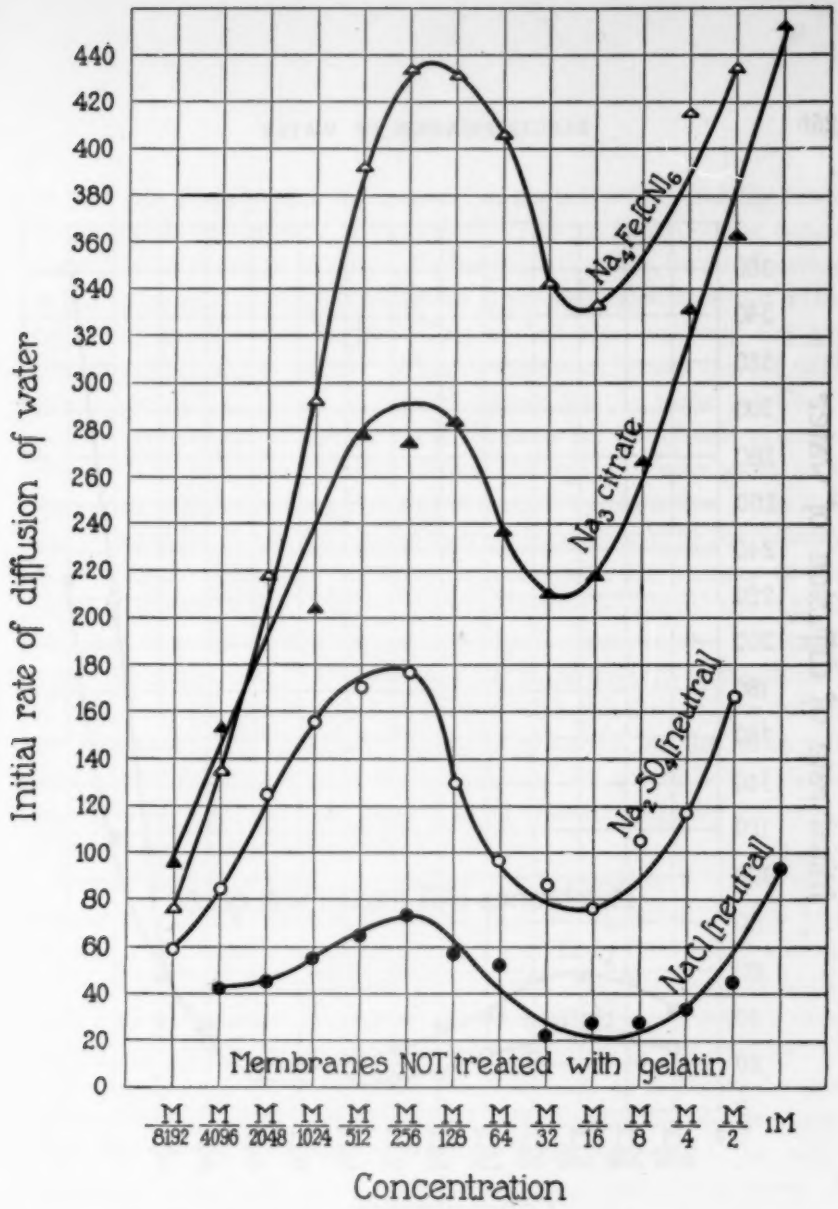


FIG. 1. Curves representing influence of concentration of solutions of different Na salts upon the initial rate of diffusion of water from pure water into solution through membranes not treated with gelatin. The curves are essentially the same as those obtained with collodion membranes which had been treated with gelatin.[3] Abscissæ are the logarithms of concentration, ordinates the rise of height of level of solution in 20 minutes. Inside of collodion bag salt solution, outside $H_2O$.

[3] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 177, Fig. 3.

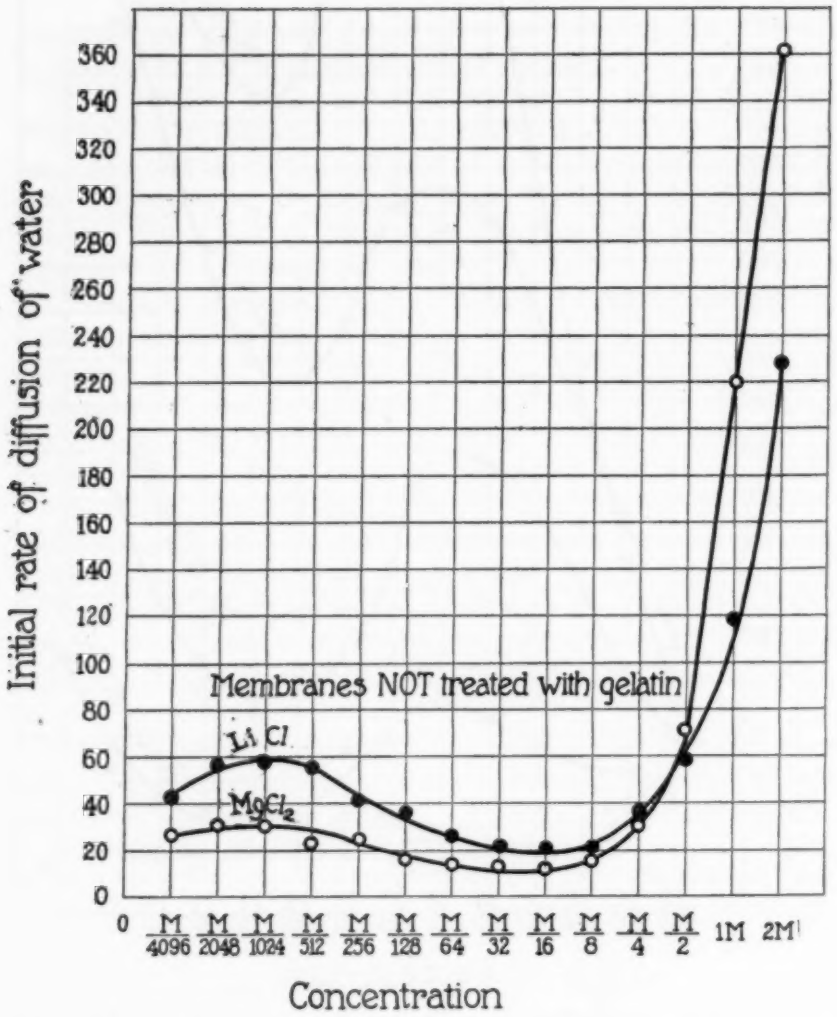


FIG 2. Same curves as in Fig. 1 for LiCl and $MgCl_2$. Curves identical for membranes treated and not treated with gelatin.

at first rise sharply with an increase in concentration and the more so the higher the valency of the anion. The maximum of the curves is reached at about M/256, then the curves fall with a further increase in the concentration until a minimum is reached again at about M/16, and then another rise begins. This second rise expresses the gas pressure effect of the solute. When we use membranes treated with gelatin, we get a similar system of curves for the same solutions,[1] and the explanation of the curves is the same for both kinds of membrane. The particles of water diffuse through the membrane as if they were positively charged, being attracted by the anion of the salt and repelled by the cation, the attraction increasing with the valency of the anion. That the repulsion increases with the valency of the cation is shown in Fig. 2 where the lower curve represents the rate of diffusion of water in 20 minutes into $MgCl_2$ solutions and the upper curve the initial rate of diffusion of water in 20 minutes into solutions of LiCl through membranes not treated with gelatin. These curves are also practically identical with those obtained for the same salt solutions when the collodion membrane had previously been treated with gelatin.[1] Hence Rule 1 holds in all essentials equally for membranes treated and not treated with gelatin.

The situation is altogether different for solutions of those electrolytes whose influence is described in Rule 2; namely, acids and neutral or acid solutions of salts with trivalent or tetravalent cation.

When we separate solutions of different concentrations of $Al_2Cl_6$ from $H_2O$ by collodion bags treated with gelatin, water diffuses very rapidly into the solution and the level of liquid in the manometer rises steeply with an increase in concentration, as is shown by the upper curve in Fig. 3. Water is negatively charged and is powerfully attracted by the trivalent cation Al. When we repeat the same experiment with membranes *not* treated with gelatin (lower curve of Fig. 3), we notice that in that range of concentrations of $Al_2Cl_6$ where the diffusion is determined chiefly (or exclusively) by electrical forces no rise occurs until the concentration of the solution of $Al_2Cl_6$ is about M/64; at about this concentration the gas pressure effect of solutions of cane sugar begins to be noticeable. It, therefore, looks as if solutions of $Al_2Cl_6$ showed no electrical but only the gas pressure effect when separated from pure water by a membrane not treated with

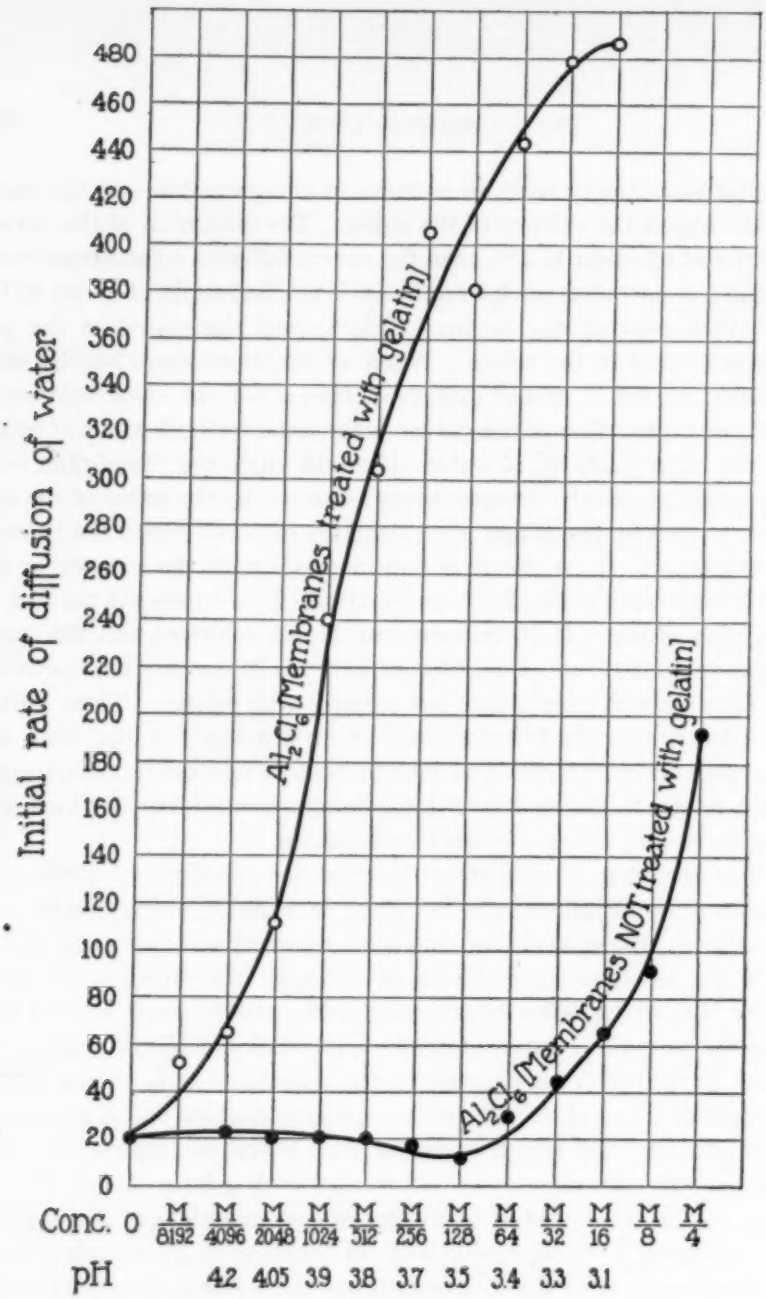


FIG. 3. Showing difference in osmotic behavior of collodion membranes treated and not treated with gelatin. Membranes separating solutions of $Al_2Cl_6$ from pure water. Upper curve, initial rate of diffusion of water (first 20 minutes) into solution through membranes treated with gelatin; lower curve, diffusion of water during the first 20 minutes into same solution of $Al_2Cl_6$ through membranes *not* treated with gelatin. In second case only the gas pressure effect of solution is apparent, while electrical attraction of water by solution is lacking.

gelatin. We shall see later that the electrical effect in the lower range of concentrations of $Al_2Cl_6$ is not lacking, but that the low ordinates are due to the action of the trivalent cation. Curves for the diffusion of water into solutions of $LaCl_3$ (Fig. 4), which are nearly neutral, show the same difference as those just described for $Al_2Cl_6$ solutions. When $LaCl_3$ solutions are separated from water by membranes not treated with gelatin, the ordinates (lower curve in Fig. 4) are low in the region of the electrical effect, while they rise steeply in the same region when the membranes have been treated with gelatin (upper curve in Fig. 4).

The difference in the osmotic behavior of the two kinds of membranes is still more striking when we use solutions of acids. When we separate solutions of strong acids (*e.g.* HCl, $HNO_3$, $H_2SO_4$) from distilled water by collodion membranes previously treated with gelatin, we notice no rise but only a drop (Fig. 5)—negative osmosis—which commences for $H_2SO_4$ and $H_3PO_4$ at a concentration of about M/256. In the experiments represented in Fig. 5, the pressure head of the solution of acids inside the bag was about 70 mm. at the beginning. It dropped in 20 minutes to about 10 mm. at concentrations of about M/8 or M/4 for $H_2SO_4$ and $H_3PO_4$ (Fig. 5). Owing to the fact that the drop is due to the repelling action of the anion of the acid upon the negatively charged particles of water, the drop in the curves is greater when the anion of the acid is bivalent or trivalent than when it is monovalent. When we separate various concentrations of the same acids from pure water by membranes *not* treated with gelatin we get results of an altogether different order (Fig. 6). Instead of negative osmosis we notice a powerful positive osmosis, *i.e.* a rapid diffusion of water into the solution, and the acids behave almost like the sodium salts with the same anion. By comparing Fig. 1 and Fig. 6 the reader will notice the same steep rise of the curves until the concentration is about M/256; this rise is more considerable in the case of $Na_2SO_4$ and $H_2SO_4$ than in the case of NaCl and HCl. In the case of these two salts and acids, the rise is followed by a drop, until for the acids at M/32 and for the salts at M/16 the gas pressure effect of the solution commences. The drop is not noticeable in the case of $H_3PO_4$.

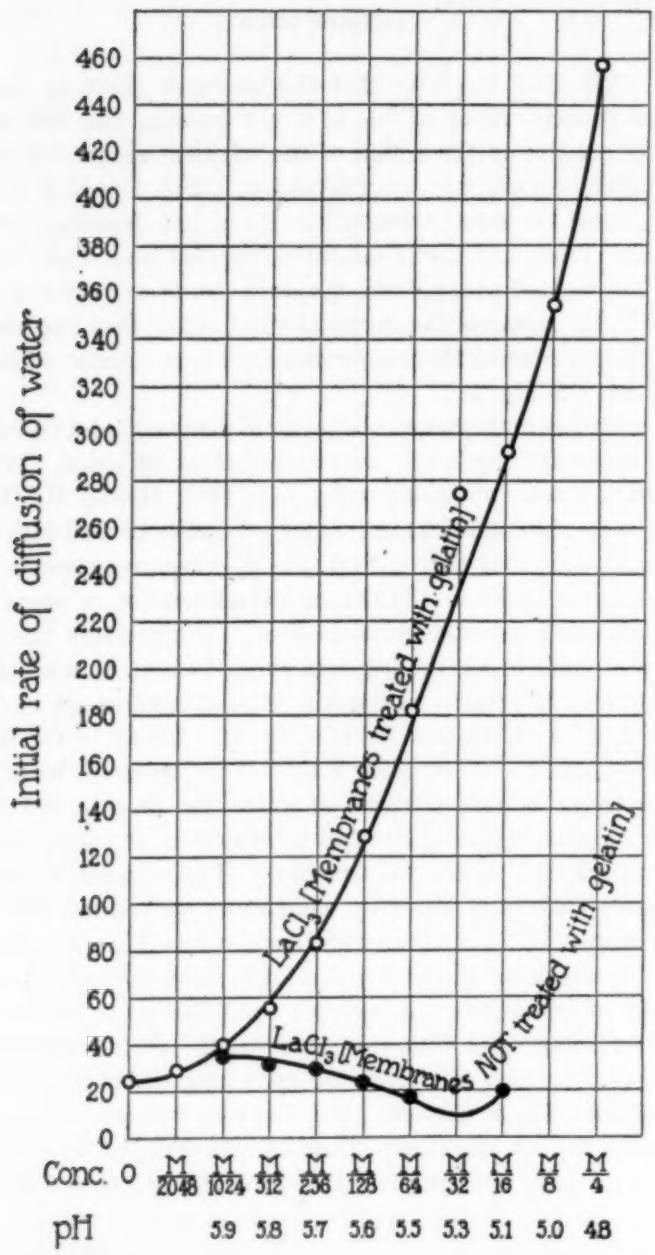


FIG. 4. Showing the same difference of membranes treated and not treated with gelatin for solutions of $LaCl_3$.

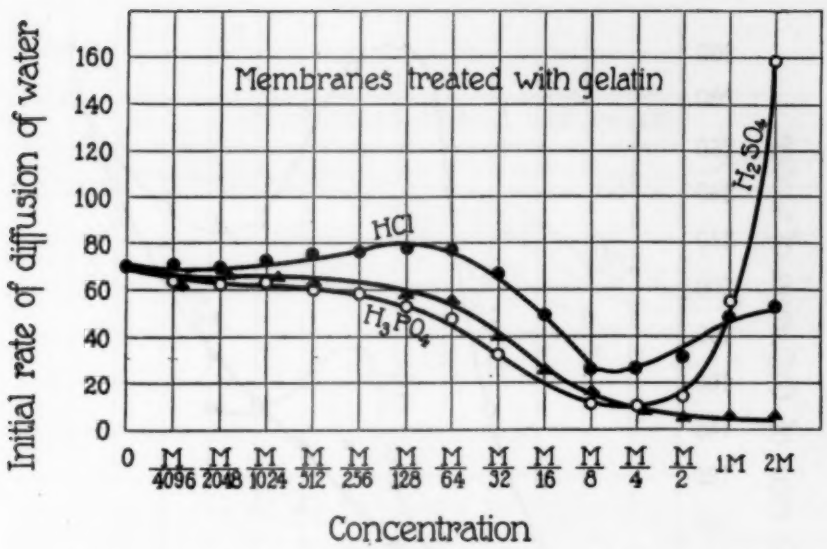


FIG. 5. Negative osmosis when acids (HCl, $H_2SO_4$, $H_3PO_4$) are separated from water by membranes treated with gelatin.

Fig. 7 gives the curves for several other acids when the membranes are treated with gelatin and Fig. 8 the curves for the same acids when the membranes are *not* treated with gelatin. When the membranes are *not* treated with gelatin the curve for oxalic acid (Fig. 8) becomes almost like that of $Na_2SO_4$ in Fig. 1; while the same acid shows the phenomenon of negative osmosis (Fig. 7) when the membrane has received a gelatin treatment previous to the experiment.

II.

What causes this profound difference in the osmotic behavior of collodion membranes according to whether they have or have *not* received a previous treatment with gelatin? It is so customary in biology to explain obscure phenomena by the assumption of variations of permeability that the writer felt it necessary to test the possibility of such an explanation in this case.

The phenomenon of positive osmosis for acid when the collodion bag separating the acid from pure water was not treated with gelatin, and the opposite phenomenon of negative osmosis when the collodion

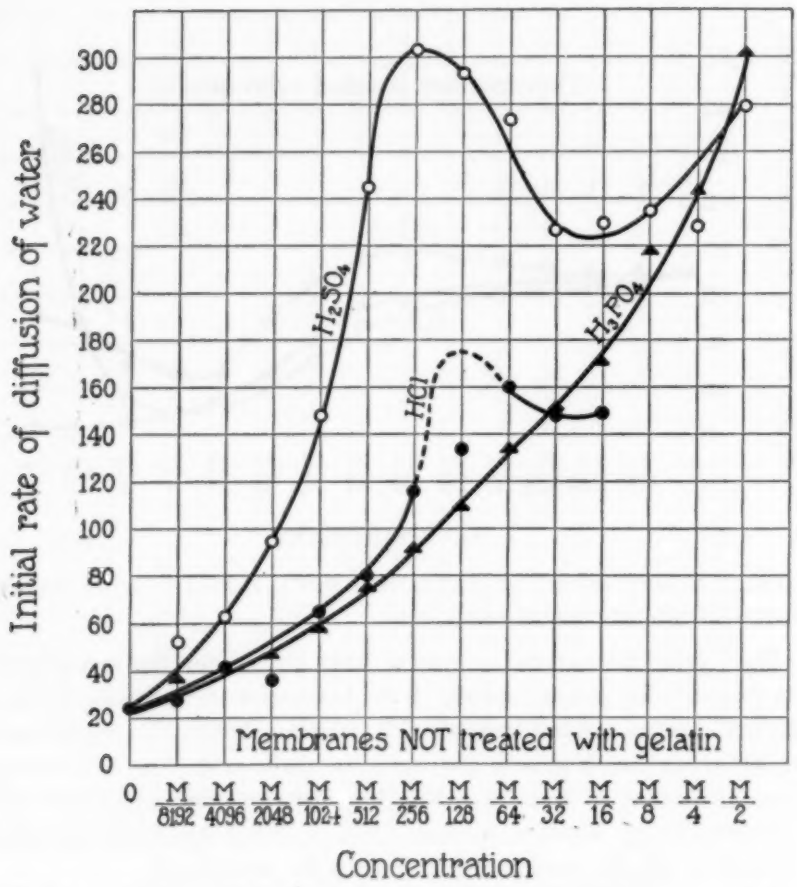


FIG. 6. Positive osmosis when the same acids as in Fig. 5 are separated from water by membranes *not* treated with gelatin. Curves similar to those obtained with sodium salts of the same anion (Fig. 1).

bag separating the acid had been treated with gelatin, suggested that the gelatin treatment had rendered the collodion bag more permeable for acid. It was, therefore, expected that titration of the contents of the bag at the beginning and after 20 minutes would show a greater diminution of the concentration of acid inside the bag when the bag had been treated with gelatin than when it had received no such treatment. The test, however, disproved the correctness of this explanation. *The rate of diffusion of acid from solution into the distilled water was practically the same* for collodion membranes treated and not treated with gelatin, as Table I shows.

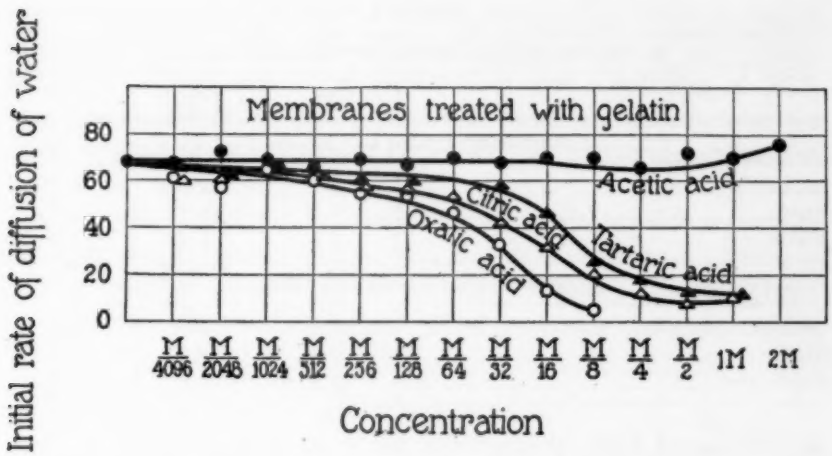


FIG. 7. Negative osmosis when solutions of acids (tartaric, citric, and oxalic) are separated from water by membranes treated with gelatin. The weak acetic acid behaves practically like a non-electrolyte.

The table shows that the amount of acid which diffused out from the collodion bag into the distilled water was (within the limits of accuracy of these experiments) the same for membranes treated and *not* treated with gelatin. Oxalic acid diffused a little more slowly than hydrochloric acid, but the rate of diffusion for each acid is approximately in direct proportion to its concentration. The fact that the same acids caused positive osmosis when the membranes were *not* treated with gelatin and negative osmosis when they were treated cannot be ascribed to differences in the permeability of the two types of collodion membranes.

Since solutions of $Al_2Cl_6$ and of $LaCl_3$ caused a vigorous diffusion of water from solvent into solution when the collodion membrane was treated with gelatin, but not when the membrane had *not* received a gelatin treatment, the idea suggested itself that the collodion membranes not treated with gelatin were so permeable for the molecules of the salt that an increase in volume of the solution by a diffusion of water into it was impossible. Titration experiments with $Al_2Br_6$ proved, however, that there was only a slight difference in the rate

TABLE I.

| Loss of cc. of 0.01 N acid for 10 cc. of solution in 20 min. | | | |
|---|---|---|---|
| | N/32 | N/64 | N/128 |
| Membranes treated with gelatin. | | | |
| HCl | 10.2 | 5.0 | 2.3 |
| $H_2SO_4$ | 9.7 | 4.7 | 2.2 |
| Oxalic acid | 7.8 | 4.0 | 1.9 |
| Membranes not treated with gelatin. | | | |
| HCl | 11.0 | 4.9 | 2.3 |
| $H_2SO_4$ | 9.8 | 4.1 | 1.7 |
| Oxalic acid | 8.1 | 3.4 | 1.6 |

of diffusion of $Al_2Br_6$ through the two types of membranes; the non-treated membrane being slightly more permeable. The difference was, however, much too small to account for the difference in the osmotic behavior of the two types of membranes.

It was, therefore, necessary to look for another explanation in the different osmotic behavior of the two types of membranes. It seemed these differences would find their explanation if it could be proved that water diffuses through collodion membranes not treated with gelatin in the form of positively charged particles even in the presence of acids or salts with trivalent or tetravalent cation. For this purpose the method of electrical endosmose was applied. Identical solutions of $LaCl_3$ were put inside and outside a collodion bag not treated with gelatin and a current (of about 4.8 milliamperes and about 90 volts) was sent through the solution. The water migrated to the cathode, showing that its particles were positively charged in the presence of $LaCl_3$. When the same experiment was repeated with membranes previously treated with gelatin it was found that water is negatively charged in the presence of solutions of salts with trivalent cation. It was furthermore possible to show that in the presence of acids water diffuses through membranes not previously treated with gelatin in the form of positively charged particles.

We can now understand why it is that water cannot diffuse through collodion membranes not treated with gelatin into solutions of salts with trivalent cation, *e.g.* $AlCl_3$ or $LaCl_3$ (in the region of electrical effect), since these particles of water carrying a positive charge are

repelled by the trivalent cation. We understand also why the initial rate of diffusion of water through membranes *not* treated with gelatin is greater into a solution of $H_2SO_4$ than into a solution of HCl, since the positively charged particles of water are attracted by the anion of the acid and the more powerfully the higher the valency of the anion.

A very simple test will demonstrate that the water diffuses through collodion membranes *not* treated with gelatin in the form of positively charged particles even in the presence of M/1,000 acid. We know from Rule 1 (at the beginning of this paper) that positively charged particles

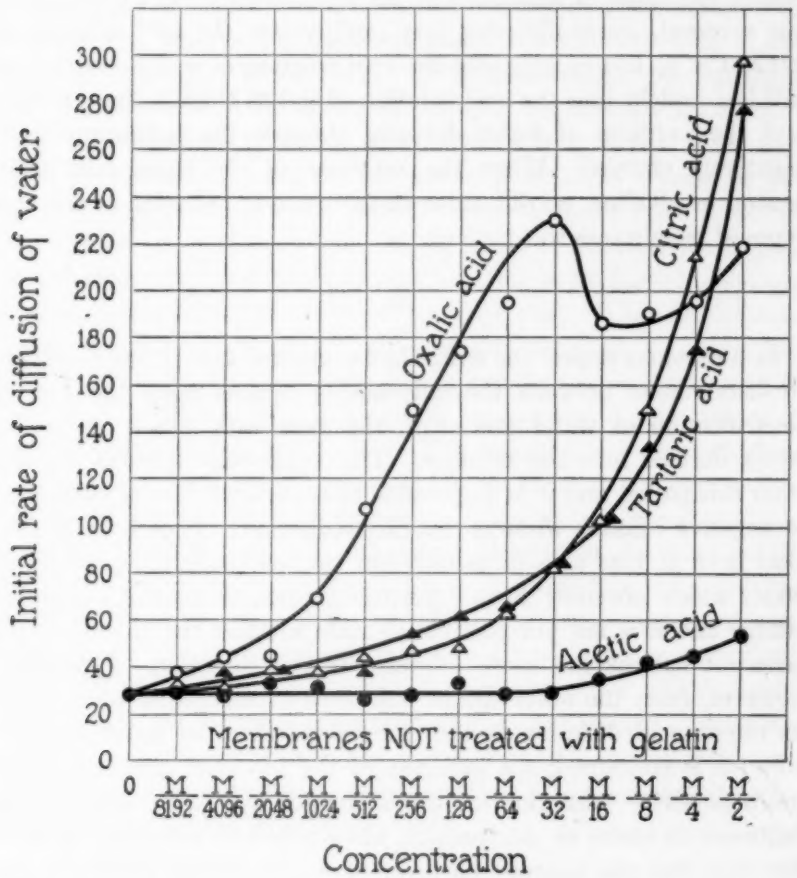


FIG. 8. Positive osmosis when solutions of the same acids as in Fig. 7 are separated from water by membranes *not* treated with gelatin.

of water are attracted more powerfully by $Na_2SO_4$, less powerfully by NaCl, and still less powerfully by $CaCl_2$. Hence when we make M/128 solutions of these three salts acid through the addition of $HNO_3$, and if it is true that water diffuses through the membranes not treated with gelatin in the form of positively charged particles in the presence of strong acid, it should diffuse more rapidly into $Na_2SO_4$, less rapidly into NaCl, and least rapidly into $CaCl_2$. M/128 solutions of these three salts were brought to a pH of 2.9 through the addition of $HNO_3$, and the experiment turned out as the theory demands. When the same experiment was made with collodion membranes treated with gelatin, the order of attraction of the three salts of pH 2.9 for water was reversed, water diffusing very rapidly into the acid solution of M/128 $CaCl_2$, less rapidly into the acid solution of M/128 NaCl, and still less rapidly into the acid solution of M/128 $Na_2SO_4$, since in this case the particles of water diffusing through the membrane were negatively charged. When the solutions of the three salts were neutral or alkaline, no difference in the osmotic behavior of the two types of membranes was noticed.

III.

In a previous paper[1] the writer had suggested that it was necessary to discriminate between the influence of cations upon the sign of electrification of water and upon the rate with which electrified water diffuses into the solution. This suggestion receives support from this paper, and it is impossible to understand the phenomenon of negative osmosis without this discrimination. Thus it is obvious that both H ions as well as trivalent cations cause the particles of water which normally have a positive charge, to assume a negative charge and it is not yet possible to state whether the H ions or the trivalent cations are more efficient in this direction. It follows, however, from the observations published in this paper that, as far as the rate of diffusion of the charged particles of water into the solution is concerned, the influence of the trivalent cations is much more powerful than that of the hydrogen ions. We express this influence in terms of electrostatic attraction and repulsion between the ions and the charged particles of water, not in order to offer thereby an explanation of this influence but merely to simplify the

presentation of the facts. With this reservation our experiments suggest that the apparent electrostatic effect of the hydrogen ions upon the motion of the electrified particles of water is small if compared with the effect of the trivalent cations; in fact it seems smaller even than that of other monovalent cations.

When acid is put into collodion bags *not* treated with gelatin, we observe a rapid diffusion of water into the acid solution as if the attraction of the positively charged water by the anion of the acid was very strong while its repulsion by the H ion was weak. When we put acids into collodion bags treated with gelatin we observe negative osmosis; *i.e.*, slight attraction of the negatively charged particles of water by the positive hydrogen ion, and a powerful repulsion of the water by the anion of the acid. In all these cases, the apparent electrostatic effect of the hydrogen ion upon the charged particles of water seems smaller than the electrostatic effect of any other ion. When we compare the electrostatic effect of trivalent cations on the rate of diffusion of water, we notice that it is much more powerful than that of the hydrogen ion. When water is negatively charged, it is attracted very powerfully by $Al_2Cl_6$ as well as by $Al_2(SO_4)_3$ though naturally more by the former than by the latter. While in the case of acids the $SO_4$ ion has a more powerful influence than the H ion, the electrostatic effect of a trivalent cation cannot be overcome by the effect of a bivalent anion. It can only be equalled by the influence of a trivalent anion; *e.g.*, the citrate. This comparatively weak electrostatic effect of the H ion is responsible for the phenomenon of negative osmosis observed in the case of acids. When we use membranes in which the electrified water is negatively charged in the presence of acid the attractive effect of the H ion is so small that the repulsive effect of the anion prevails in the critical range of concentrations, where the drop in the curve occurs. As a consequence no water can diffuse into the solution and the volume of the latter will diminish on account of the diffusion of solute into the pure solvent. Since we notice also negative osmosis in the case of $Ba(OH)_2$ and $Ca(OH)_2$, we conclude that the electrostatic effect of the OH ion on the movable stratum of the double layer is also small.

IV.

Gelatin is not the only substance which causes a reversal of the sign of the electrification of the particles of water diffusing through the collodion membrane in the presence of comparatively low concentrations of acid or of salts with trivalent or tetravalent cations. The same effect can be produced when the collodion membrane is treated with 1 per cent solutions of casein, egg albumin, blood albumin, and edestin; while treatment of the collodion membrane with 1 per cent solutions of Fairchild's peptone, of peptone (prepared by Dr. Northrop) from egg albumin digested with pepsin and neutralized, of alanine, of "soluble starch," of "potato starch," and of 0.50 per cent agar-agar did not cause the reversal. The proteins which modify the behavior of the membrane cannot diffuse through the latter but the writer is not yet ready to state that this is the decisive factor. It is of interest that a treatment of the collodion membrane with a 0.002 per cent solution of gelatin makes the membrane already noticeably amphoteric though to a considerably smaller degree than a treatment with a 1 per cent solution of gelatin.

SUMMARY.

1. It is shown that collodion membranes which have received one treatment with a 1 per cent gelatin solution show for a long time (if not permanently) afterwards a different osmotic behavior from collodion membranes not treated with gelatin. This difference shows itself only towards solutions of those electrolytes which have a tendency to induce a negative electrification of the water particles diffusing through the membrane, namely solutions of acids, acid salts, and of salts with trivalent and tetravalent cations; while the osmotic behavior of the two types of membranes towards solutions of salts and alkalies, which induce a positive electrification of the water particles diffusing through the membrane, is the same.

2. When we separate solutions of salts with trivalent cation, *e.g.* $LaCl_3$ or $AlCl_3$, from pure water by a collodion membrane treated with gelatin, water diffuses rapidly into the solution; while no water diffuses into the solution when the collodion membrane has received no gelatin treatment.

3. When we separate solutions of acid from pure water by a membrane previously treated with gelatin, negative osmosis occurs; *i.e.*, practically no water can diffuse into the solution, while the molecules of solution and some water diffuse out. When we separate solutions of acid from pure water by collodion membranes not treated with gelatin, positive osmosis will occur; *i.e.*, water will diffuse rapidly into the solution and the more rapidly the higher the valency of the anion.

4. These differences occur only in that range of concentrations of electrolytes inside of which the forces determining the rate of diffusion of water through the membrane are predominantly electrical; *i.e.*, in concentrations from 0 to about M/16. For higher concentrations of the same electrolytes, where the forces determining the rate of diffusion are molecular, the osmotic behavior of the two types of membranes is essentially the same.

5. The differences in the osmotic behavior of the two types of membranes are not due to differences in the permeability of the membranes for solutes since it is shown that acids diffuse with the same rate through both kinds of membranes.

6. It is shown that the differences in the osmotic behavior of the two types of collodion membranes towards solutions of acids and of salts with trivalent cation are due to the fact that in the presence of these electrolytes water diffuses in the form of negatively charged particles through the membranes previously treated with gelatin, and in the form of positively charged particles through collodion membranes *not* treated with gelatin.

7. A treatment of the collodion membranes with casein, egg albumin, blood albumin, or edestin affects the behavior of the membrane towards salts with trivalent or tetravalent cations and towards acids in the same way as does a treatment with gelatin; while a treatment of the membranes with peptone prepared from egg albumin, with alanine, or with starch has no such effect.

# INFLUENCE OF THE CONCENTRATION OF ELECTROLYTES ON SOME PHYSICAL PROPERTIES OF COLLOIDS AND OF CRYSTALLOIDS.

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*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

(Received for publication, November 19, 1919.)

## I. INTRODUCTION.

When we separate neutral solutions of salts with monovalent cation from pure water by a collodion membrane, water will diffuse into the solution. The writer has shown in a preceding publication[1] that the initial rate of diffusion will at first increase with the concentration of the electrolyte, but as soon as the concentration of the latter is about M/256, the initial rate of diffusion of water into the solution will in the case of many electrolytes diminish with a further increase in the concentration of the electrolyte until at a concentration varying between M/32 and M/8 (according to the nature of the electrolyte) a minimum is reached. This phenomenon is due to the influence of the ions on the electrification and rate of diffusion of water through the collodion membrane. In the presence of neutral solutions of salts with monovalent or bivalent cation, water diffuses through the collodion membrane as if its particles were positively charged and as if they were attracted by the anion and repelled by the cation of the electrolyte with a force increasing with the valency of the ion. With low concentrations of electrolytes the attractive action of the anion upon the positively charged particles of water prevails over the repulsive force of the cation, while, when the concentration exceeds a certain value, which for a number of salts is about M/256, the repelling force of the cations of the electrolyte upon the positively charged particles of the water increases more rapidly than the attractive force of the anions. This idea is supported by the fact that the addition of salts with

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173.

bivalent cation causes a more rapid drop than the addition of a salt with monovalent cation. These phenomena bear so striking a resemblance to the action of the concentration of electrolytes upon the osmotic pressure, the swelling, and other properties of colloids that a discussion of the similarity may seem of interest.

It was found by Pauli[2] that the addition of a little acid to blood albumin which had been dialyzed for weeks (and which was therefore approximately isoelectric) caused an increase in the viscosity of the protein, which at first was the greater the more acid was added. Very soon, however, a point was reached where the addition of more acid caused again a diminution in the viscosity. The same phenomenon occurs when acid is added to isoelectric gelatin. The addition of a slight amount of acid causes an increase in the osmotic pressure until finally a point is reached where the further addition causes a diminution (Fig. 1). The increase in the osmotic pressure of isoelectric gelatin when a slight quantity of HCl is added, is due to the formation of gelatin chloride, but the depressing effect of the addition of an excess of acid is not so easy to explain. According to Pauli we should ascribe it to the diminution of the degree of electrolytic dissociation of protein chloride due to the increase in the concentration of the common anion Cl. The writer's measurements of conductivity do not support this idea.[3] Another suggestion made by colloid chemists is that the addition of more acid causes an aggregate formation of the gelatin particles and therefore a diminution of osmotic pressure. This suggestion rests only on the phenomenon which it is supposed to explain, but it may, nevertheless, be correct. If so, it remains to be explained why an increase in the concentration of electrolytes causes a formation of aggregates.

In a preceding publication[4] the writer has shown that a 1 per cent solution of gelatin-acid salt, *e.g.* gelatin chloride or gelatin citrate, etc., has its maximal osmotic pressure when the pH is about 3.4 or 3.3. When to gelatin chloride of this pH acid or neutral salt is added, the osmotic pressure (as well as the swelling, viscosity, etc.) falls and the more so the more acid or salt has been added. When we add

[2] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 223.
[3] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 483, 559.
[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 559.

alkali, the osmotic pressure falls also, but in this case a complication arises, since the addition of NaOH to a gelatin-acid salt causes a neutralization of the acid and the gradual transformation of the gelatin-acid salt into isoelectric gelatin which has a minimal osmotic pressure.

A 1 per cent solution of metal gelatinate, *e.g.* Na gelatinate, has its maximal osmotic pressure at a pH of about 8.4. When to a 1 per cent solution of metal gelatinate of this pH alkali or neutral salt is added, the osmotic pressure (as well as the swelling and the viscosity) is diminished. When acid is added the same phenomenon occurs but for another reason since the addition of acid lowers the pH and transforms gelatin salt into isoelectric gelatin. We will first discuss the action of electrolytes on the osmotic pressure of metal gelatinate.

### *II. Water Charged Positively.*

Doses of 1 gm. each of commercial, finely powdered gelatin are rendered isoelectric, melted, and made up into 1 per cent solutions of gelatin by adding enough NaOH and water to make the volume 100 cc. The amount of NaOH contained in 100 cc. of each solution varied and it was for different solutions 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of M/4 NaOH. Part of this NaOH combined with the gelatin, forming Na gelatinate; the rest remained free. As shown in a preceding paper, there exists a definite chemical equilibrium between the Na gelatinate formed, the isoelectric gelatin, and the hydrogen ion concentration. If the alkali of such a solution is neutralized by an acid (*e.g.* $CO_2$) or if it is allowed to diffuse out from the solution, the equilibrium is disturbed and some of the metal gelatinate will be transformed into non-ionogenic (isoelectric) gelatin.

These gelatin solutions containing different amounts of NaOH were put into collodion bags. The latter were closed with a rubber stopper which was perforated by a glass tube with a bore of 2 mm. in diameter which served as a manometer to measure the osmotic pressure of the solutions. These bags were put into beakers containing 350 cc. of a solution of NaOH which in each case had the same amount of NaOH in 100 cc. solution as was originally added to the gelatin solution which it surrounded. Thus the 1 per cent solution of Na

gelatinate which had 6.4 cc. of M/4 NaOH in 100 cc. was dipped into a beaker with water which had 6.4 cc. of M/4 NaOH per 100 cc. of $H_2O$. Since a small part of the NaOH inside the collodion bag had combined with the gelatin, the concentration of NaOH in the outside solution was at first slightly greater than that inside, and as a consequence some NaOH diffused from the outside into the bags. Part of the NaOH in the outside solution was gradually neutralized by

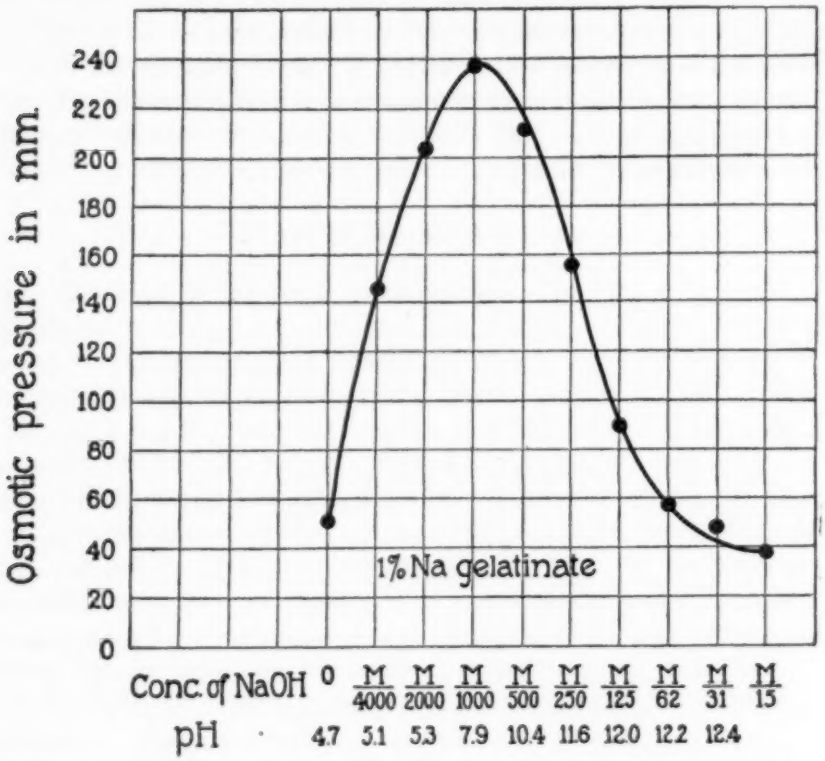



FIG. 1. Curve of osmotic pressure of 1 per cent Na gelatinate solutions contained in collodion bags and surrounded by solutions of NaOH of the same concentration as that in which the isoelectric gelatin was dissolved. Abscissæ are the logarithms of concentration of NaOH in which the isoelectric gelatin was dissolved; below is given the pH of the gelatin solution at the conclusion of the experiment (4½ hours after beginning). The osmotic pressure rises steeply at first until the pH is about 8.4, and then when more NaOH is contained in solution the osmotic pressure falls again equally steeply with a further increase in concentration of NaOH added.

the absorption of $CO_2$ from the air and this diminished the concentration of NaOH in the outside and, as a consequence, also in the inside solution.

Fig. 1 represents the osmotic pressure reached after about 5 hours in the various solutions. (At this time the permanent osmotic pressure is generally attained when the solution undergoes no further chemical changes.) The abscissæ of Fig. 1 are the logarithms of the concentration of the NaOH. The row of figures below the figures for the concentration of NaOH is the pH as found in each gelatin solution at the end of the experiment. The ordinates are the osmotic

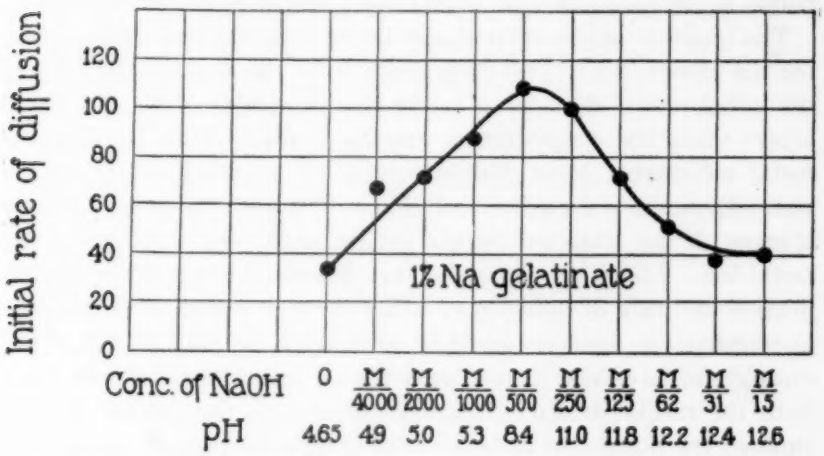



FIG. 2. Curve of initial rate of diffusion of water into 1 per cent gelatin dissolved in the same concentrations of NaOH as those used in Fig. 1, measured by rise of level of liquid in manometer 30 minutes after beginning of experiment. Abscissæ are the concentrations of NaOH, ordinates are the rise of level of liquid in manometer after 30 minutes. The curve is similar to the curve of osmotic pressure. Maximum at pH = 8.4.

pressures expressed in mm. of a column of 1 per cent gelatin solution. The maximum should have been at a pH of about 8.4 (between M/1,000 and M/500). The figure shows that the osmotic pressure rises first rapidly with an increase in the pH and falls equally rapidly beyond a pH which (from other experiments) we judge to be about 8.4. At a pH of 12.2 the curve is almost as low as it is for isoelectric gelatin.

Fig. 2 shows the influence of the concentration of NaOH upon the initial rate of diffusion of water into the solutions of Na gelatinate. The ordinates represent the height to which the liquid had risen in the manometer 30 minutes after commencement of the experiment. The abscissæ are again the logarithms of the concentration of the NaOH and the pH is that found after 30 minutes. The reader will notice that the critical points in this curve coincide with the critical points in Fig. 1. The curve rises steeply until pH = 8.4 when the maximum is reached, and falls equally steeply until a pH = 12.2 is reached. We have given the reason for this coincidence of the critical points in the curves of Figs. 1 and 2 in a preceding paper.[5]

The question arises, what causes the drop of the two curves when the pH exceeds 8.4? This drop seems to be the same as the drop in the initial rate of diffusion of water into a solution of $Na_2SO_4$ which occurs when the concentration exceeds M/256. When we separate metal gelatinates from distilled water by a collodion membrane, water diffuses into the gelatin solution as if its particles were positively charged; being attracted by the gelatin anion and repelled by the metal ion. Metal gelatinates behave therefore towards the electrification and rate of diffusion of water through collodion membranes like neutral or alkaline solutions of $Na_2SO_4$ or $Na_4Fe(CN)_6$. The analogy can be carried further, since the addition of little salt depresses both the rate at which water will diffuse into the gelatin solution through the membrane as well as the permanent osmotic pressure of the solution.

Fig. 3 shows the rapid fall in the permanent osmotic pressure of a 1 per cent solution of Na gelatinate with a pH of about 8.4 to which various concentrations of a salt, KCl, $K_2SO_4$, $K_3$ citrate, and $CaCl_2$, are added. The amounts of salts contained in 100 cc. of gelatin solution were 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of M/4. These solutions were put into the collodion bags described, and the collodion bags were put into beakers containing the same salt in the same concentration which had been added to the gelatin solution. Fig. 3 gives the rise in the manometer after 30 minutes and Fig. 4 the osmotic pressure after 6 hours. The curves show the rapid drop

[5] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 87.

of the initial rate of diffusion, as well as of the permanent osmotic pressure with the increase in the concentration of the salt added. The drop is more rapid when Ca is added than when K is added, as it should be on account of the fact that the particles of water diffusing through the membrane are positively charged.

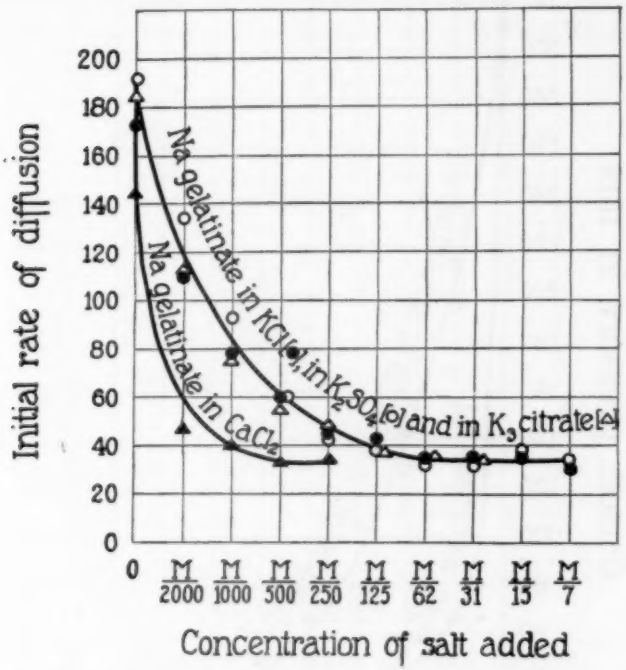



FIG. 3. Depressing effect of the addition of neutral salt to a 1 per cent solution of Na gelatinate of pH about 8.4 upon the initial rate of diffusion of water into the solution during 30 minutes. Abscissæ are the concentration of salt added, ordinates the height of level of liquid in manometer after 30 minutes. The depressing effect of Ca is greater than that of K.

Figs. 5 and 6 show that the depressing effect of the addition of alkali is the same as that of the addition of neutral salt with the same cation, $Ca(OH)_2$ causing a greater depression than KOH. This contradicts the statement current in the literature of colloid chemistry that salts lower and that alkalies raise the osmotic pressure of gelatin solutions.

The influence of the concentration of electrolytes upon the electrification and rate of diffusion of water into gelatin solutions is similar

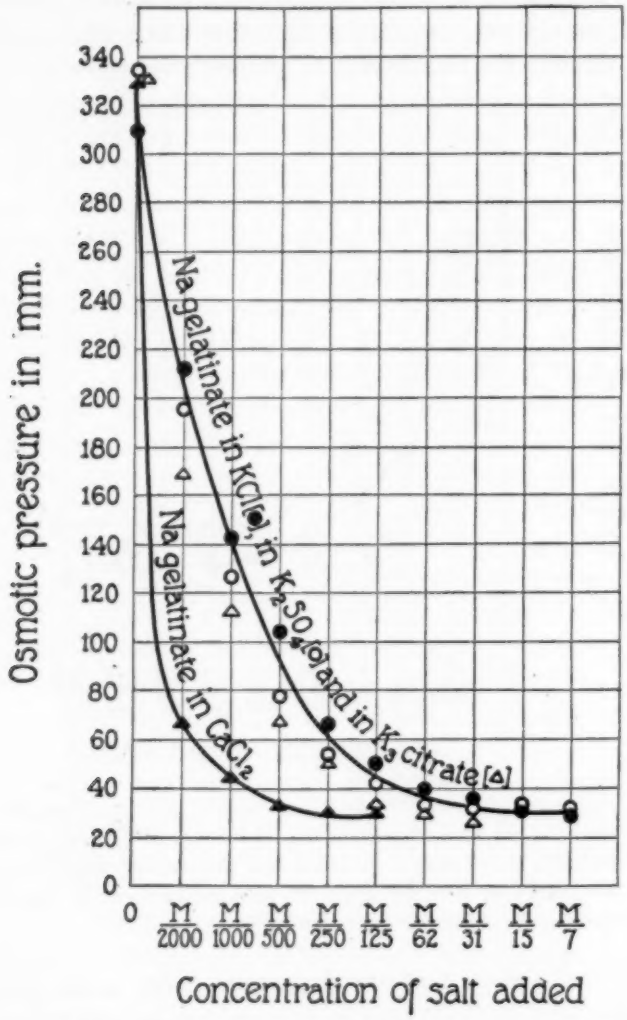



FIG. 4. Depressing effect of the addition of salts to 1 per cent Na gelatinate of pH = 8.4 upon osmotic pressure of solution. Ca depresses more than K.

to the influence of the concentration of electrolytes when we substitute a neutral salt like $Na_2SO_4$ or $Na_4Fe(CN)_6$ for the solution of metal

gelatinate. It may be well to point out this analogy by making the experimental methods in both cases as much alike as possible.

To solutions of M/256 $Na_2SO_4$ were added the same concentrations of salt as in the gelatin experiments; namely, 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of M/4 salt in 100 cc. of solution. These solutions were put into collodion bags and the latter were put into the solutions of the same salts as those in which the $Na_2SO_4$ was made

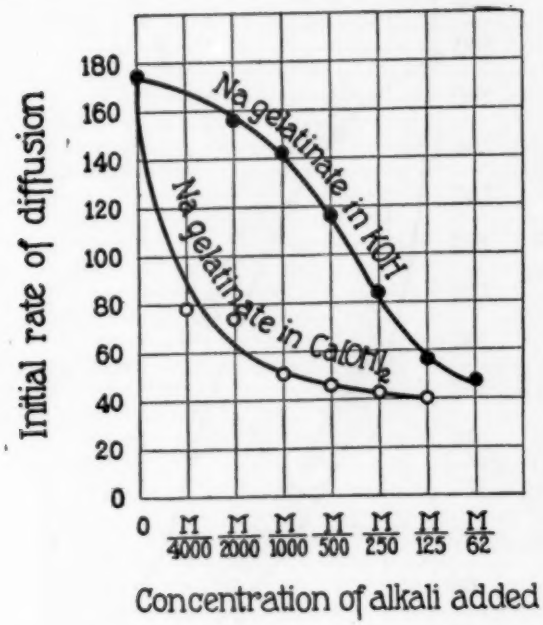



FIG. 5. Depressing effect of the addition of alkali to 1 per cent Na gelatinate of pH = 8.4 upon the initial rate of diffusion of water into the gelatin solution. $Ca(OH)_2$ depresses more than KOH.

up. This means, that when 100 cc. of the solution in the collodion bag were a mixture of M/256 $Na_2SO_4$ in M/2,000 KCl, the outside solution was M/2,000 KCl (without $Na_2SO_4$), and so on. This was done to make the experiments in every point like the gelatin experiments, in which the outside solution also contained the same concentration of salt which was added to the gelatin solution. It was found that the addition of salts and of alkali depresses the initial rate of

diffusion of water into a M/256 solution of $Na_2SO_4$ in the same way as it depresses the initial rate of diffusion of water into a solution of Na gelatinate.

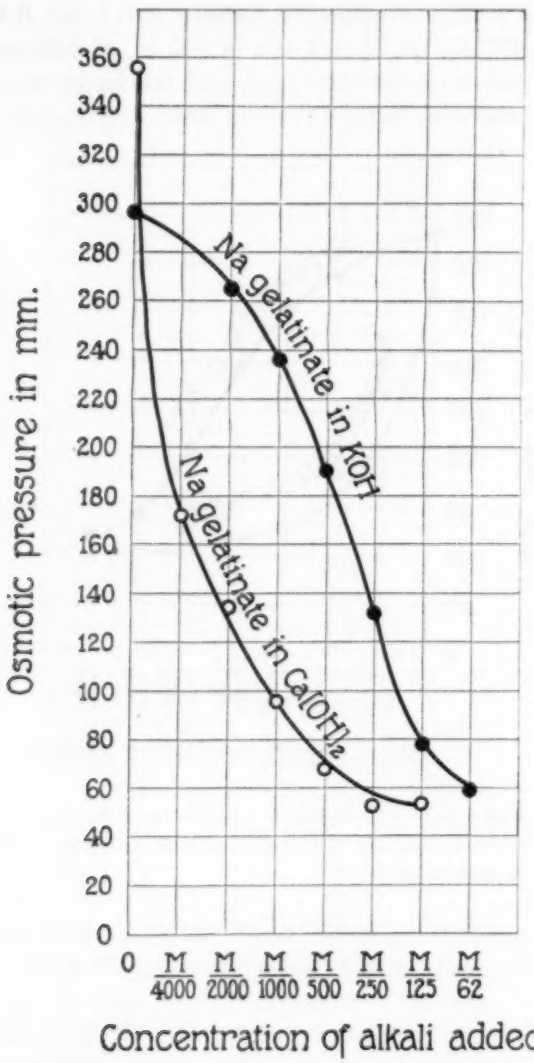



FIG. 6. Depressing effect of the addition of alkali to 1 per cent Na gelatinate of pH = 8.4 upon osmotic pressure of gelatin solution. $Ca(OH)_2$ depresses more than KOH. The effect is similar to that of addition of neutral salt.

This is illustrated by the experiments represented in Fig. 7. The abscissæ are the concentration of the salt added to the M/256 $Na_2SO_4$ solutions. The electrolytes added were KOH, KCl, and $MgCl_2$.

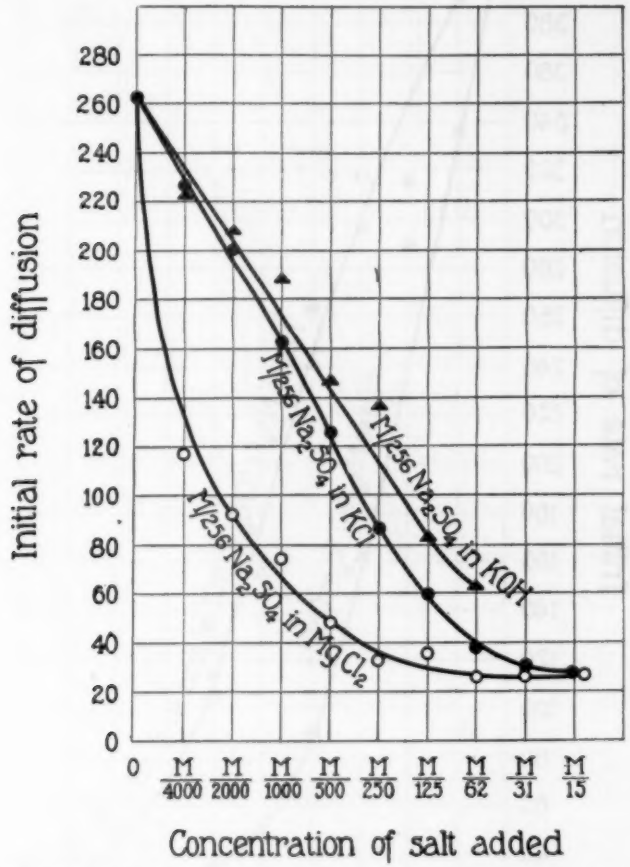



FIG. 7. M/256 $Na_2SO_4$ solutions separated from water by a collodion membrane, showing that the addition of salt or alkali to M/256 $Na_2SO_4$ causes a similar depression upon the initial rate of diffusion of water into the solution of M/256 $Na_2SO_4$ as the addition of salt to a solution of Na gelatinate. Mg depresses more than K.

There was little difference in the effect of KOH and of KCl, while the depressing effect of $MgCl_2$ was considerably greater. *It should be stated that the collodion membranes used in these experiments had not been treated with gelatin.*

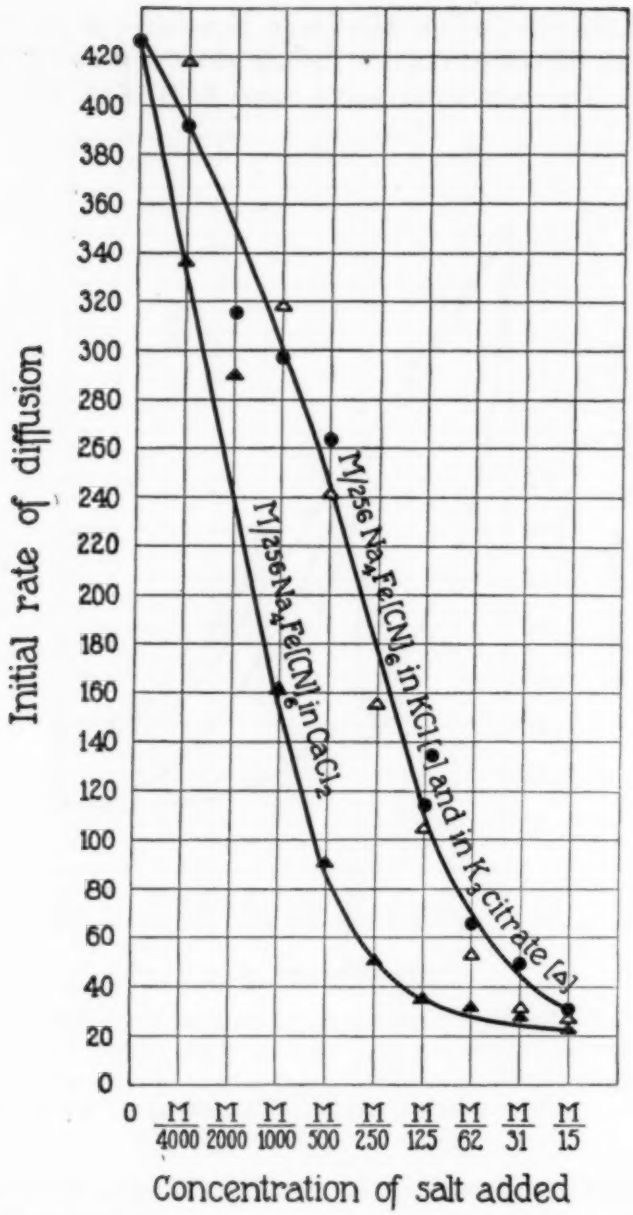



FIG. 8. M/256 $Na_4Fe(CN)_6$ separated from water by a collodion membrane, showing the depressing effect of the addition of salt to the solution upon the initial rate of diffusion of water into the solution.

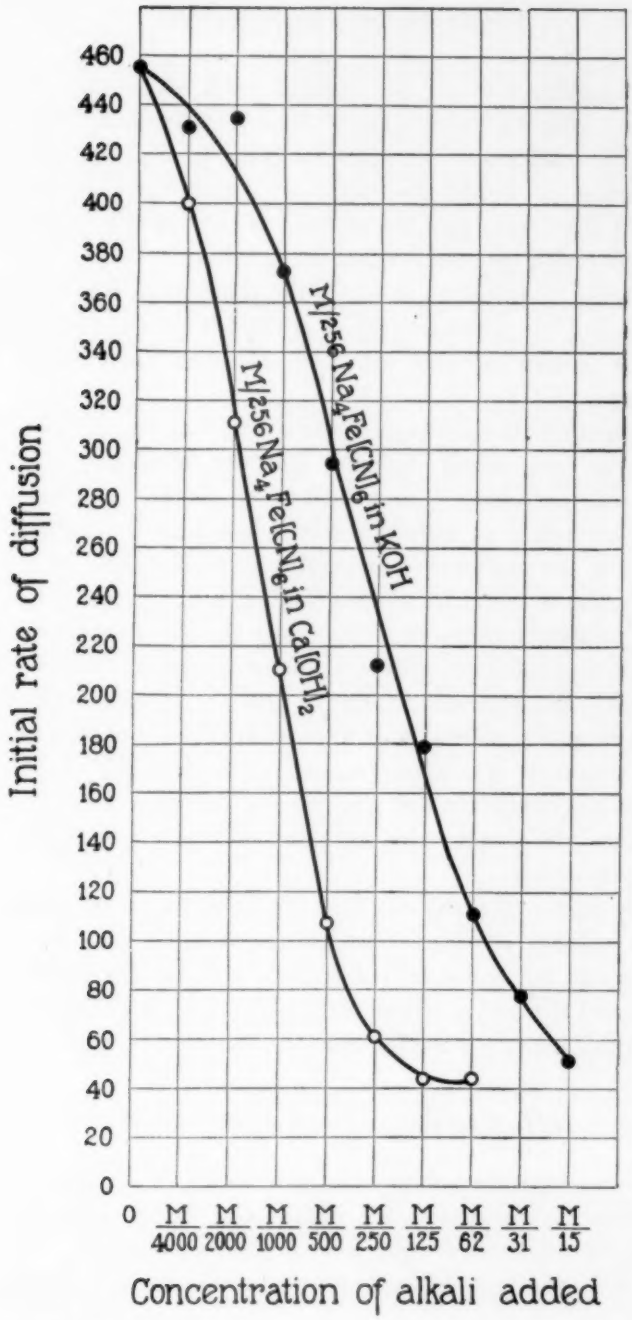



FIG. 9. Showing the same depressing effect of the addition of alkali to M/256 $Na_4Fe(CN)_6$.

These experiments can be repeated with any other neutral or alkaline salt with univalent cation. Figs. 8 and 9 show the depressing effect of the addition of neutral salts (Fig. 8) or of alkalies (Fig. 9) to a solution of M/256 $Na_4Fe(CN)_6$. Again the influence of the valency of the cation and the identity of the effects of alkalies and neutral salts with the same cation are manifest.

In all these experiments in which the particles of water were positively charged the depressing effect of the addition of salt was greater when the cation of the salt was bivalent than when it was monovalent.

Such experiments have been made with a large number of salts, all yielding the same result; namely, that the depressing effect of the addition of increasing concentrations of alkalies and neutral salts upon the attraction of water by gelatin solutions is paralleled by the influence of the addition of increasing concentrations of salts upon the attraction of water by solutions of salts, in the presence of which water is positively charged. This raises the question whether or not the depressing influence of high concentrations of electrolytes upon the osmotic pressure of gelatin is necessarily connected with the colloidal character of gelatin or whether it is based upon a much more general property of matter; namely, the influence of electrolytes upon the electrification and the rate of diffusion of water through membranes. We only wish to point out this possibility without deciding definitely.

It may suffice to point out that the influence of the concentration and the valency of electrolytes on the osmotic pressure of gelatin solutions can be demonstrated equally well for the phenomenon of swelling and the curves representing this influence are similar to those given in this paper for the influence on the osmotic pressure.

### *III. Negatively Charged Particles of Water.*

When we separate solutions of gelatin-acid salts from pure water by a collodion membrane, the particles of water diffusing through the membrane act as if they were negatively charged. That this is so can be demonstrated by experiments on electrical endosmose.

When acid, *e.g.* HCl, is added to isoelectric gelatin, part of the acid combines with gelatin to form gelatin chloride and part of the acid remains free. There is a chemical equilibrium between isoelectric gelatin, gelatin chloride, and free hydrochloric acid. As long as the pH of the gelatin chloride solution does not exceed 3.3 (*i.e.* as long as the pH varies between 4.7 and 3.3) the osmotic pressure of gelatin chloride increases with an increase in the concentration of the acid. As soon, however, as the pH reaches 3.3, the osmotic pressure of gelatin chloride diminishes again with increasing concentration of acid.

Fig. 10 may serve as an illustration. To each of a series of doses of 1 gm. of isoelectric gelatin were added 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of M/4 HCl, water was added, the gelatin melted by heating to about 40°C., and enough water was added to make the volume of the solution 100 cc., so that each solution contained 1 per cent gelatin. This solution was put into collodion bags closed by a rubber stopper perforated with a glass tube serving as a manometer. The collodion bag was put into a beaker containing 350 cc. of the same concentration of acid in water as that originally added to the gelatin. Thus the gelatin solution to which 0.2 cc. of M/4 HCl was added per 100 cc. of solution was immersed in a HCl solution containing 0.2 cc. of M/4 HCl in 100 cc. of water. This outside solution was a little more concentrated than the concentration of the free acid inside, since part of the acid added to the gelatin entered into combination with the latter. This difference was equalized by the diffusion of some of the outside acid into the gelatin solution, thus slightly lowering the original pH.

The upper curve (Fig. 10) shows that the osmotic pressure of the solution (measured after 20 hours) rises with an increase of the hydrogen ion concentration until it reaches a maximum at pH about 3.4 or 3.3, and that with a further rise in the amount of free acid the osmotic pressure of the solution falls until at pH 1.75 the osmotic pressure of the gelatin solution is almost as low as it is near the isoelectric point.

The lower curve in Fig. 10 represents the velocity of diffusion of water into the gelatin solution measured by the height of the column of liquid in the manometer after 30 minutes. The maximum and the two minima of the curve coincide with those of the upper curve

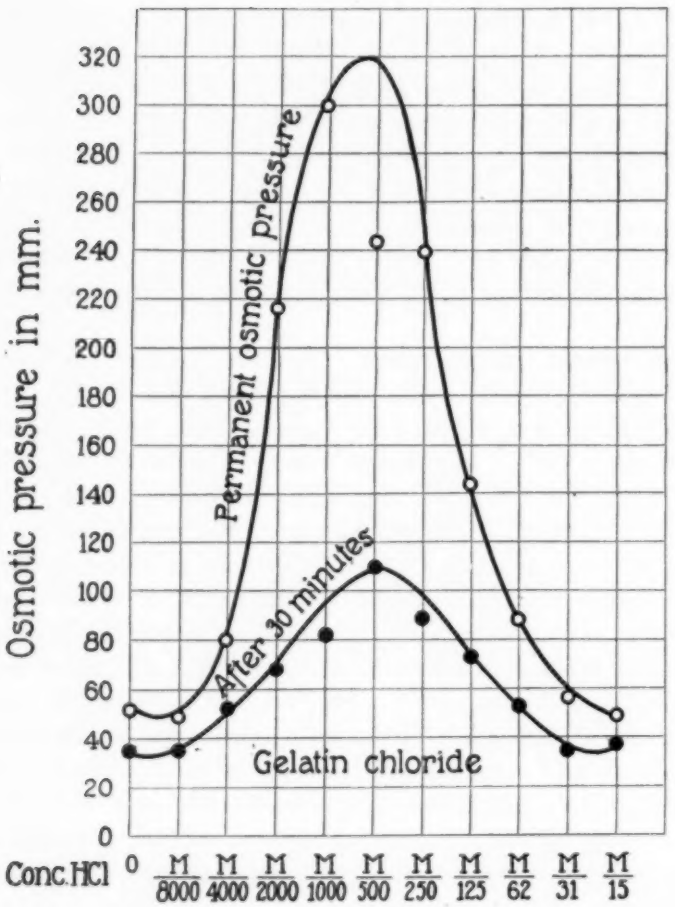



FIG. 10. 1 per cent isoelectric gelatin solutions dissolved in increasing concentrations of HCl. Abscissæ are the concentrations of HCl in which the gelatin is dissolved, with the pH at the end and at the beginning of experiment. Ordinates of upper curve represent the final osmotic pressure (after 20 hours); ordinates of lower curve represent the initial rate of diffusion of water into solution. Maximum in both cases at pH 3.3. Showing the depressing effect of the further addition of HCl when pH 3.3 is reached.

representing the permanent osmotic pressures of the various gelatin solutions.

When we select gelatin chloride with about the maximal osmotic pressure, *i.e.* gelatin chloride with a pH of 3.5, and add various

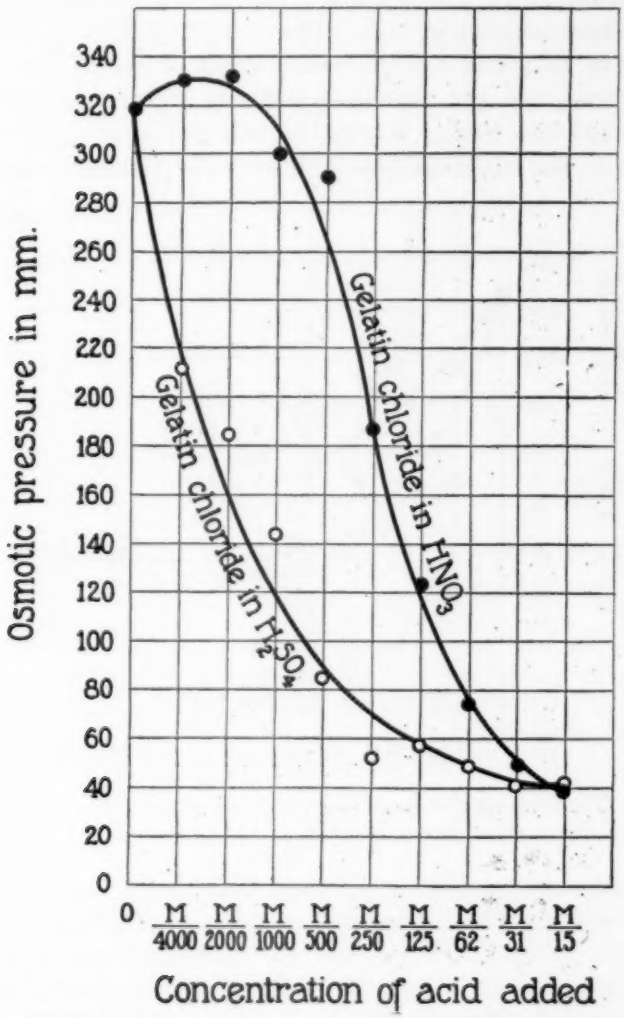



FIG. 11. Showing depressing effect of addition of $HNO_3$ and $H_2SO_4$ to 1 per cent gelatin chloride upon osmotic pressure. Depressing effect of $SO_4$ greater than that of $HNO_3$.

concentrations of another acid to it, we notice a similar depression. Fig. 11 gives the effect of the addition of different quantities of $HNO_3$ and of $H_2SO_4$ to 1 gm. of gelatin of pH 3.3; 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of M/4 acid were contained in 100 cc. of gelatin solution of pH 3.5. The outside solution contained the same concentration of acid. The depressing effect of $HNO_3$ was like that of HCl, and the depressing effect of $H_2SO_4$ was greater. Fig. 12 shows that the depressing influence of the addition of acid is manifested in a similar way in the influence of the concentration of acid upon the initial diffusion of water into the gelatin solution.

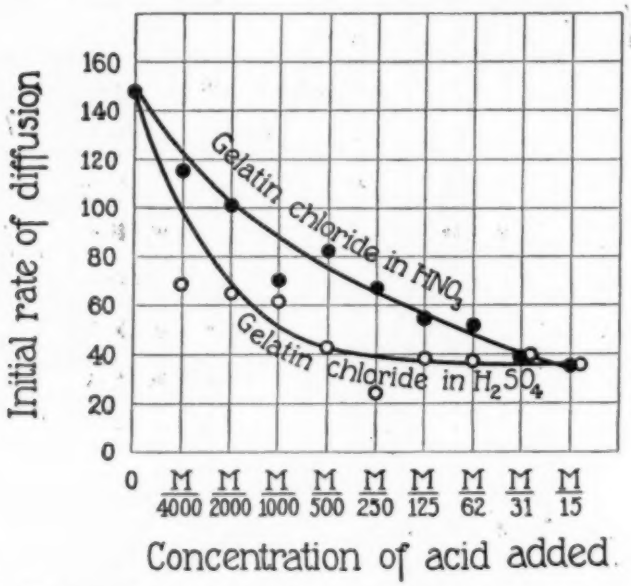
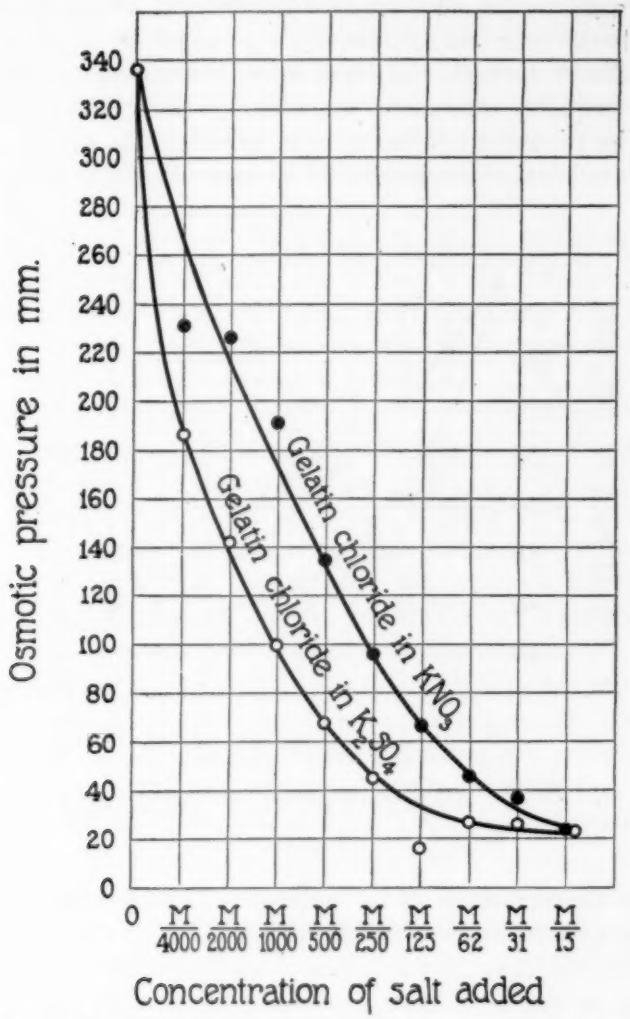



FIG. 12. The same effect of the addition of acid upon the initial rate of diffusion of water into the gelatin chloride solution.

If we add neutral salt, *e.g.* $KNO_3$ or $K_2SO_4$, to a 1 per cent gelatin solution with a pH = 3.5, taking care that the pH of the solution is not altered by the addition of salt, the depressing effect is about the same as when we add acid. To doses of 1 gm. isoelectric gelatin of pH 3.5 were added 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of M/4 $KNO_3$ or $K_2SO_4$, and enough water was added to make the volume 100 cc. The pH remained the same in all solutions.

The outside solutions had the same pH as the solution in the collodion bag and contained also the same concentration of potassium




FIGS. 13 and 14. Depressing effect of addition of salt to 1 per cent gelatin chloride pH = 3.3. $SO_4$ depresses more than $NO_3$.

salt as was added to the gelatin. The curves in Figs. 13 and 14 show that the depressing effect of an addition of $KNO_3$ is about the same as the addition of an equal amount of $HNO_3$, and that the addition

of $K_2SO_4$ has about the same depressing effect as the addition of $H_2SO_4$.

When gelatin-acid salts, *e.g.* gelatin chloride, are separated from distilled water or a salt solution of the same pH by a collodion bag, water diffuses through the membrane in the form of negatively charged particles which are attracted by the gelatin cation and repelled by the anion. When we wish to replace the gelatin solution by a crystalloidal electrolyte in the presence of which water diffuses

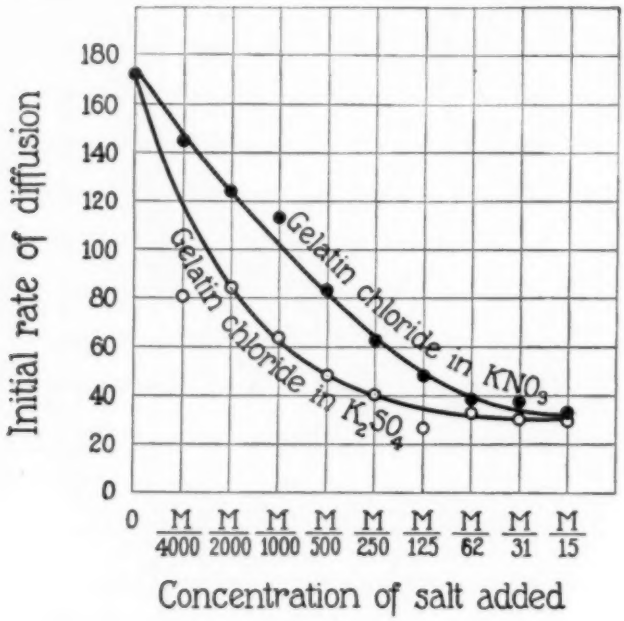



FIG. 14.

through the membrane in the form of negatively charged particles, we have to fulfill two conditions. First, we must use as electrolytes salts with trivalent (or tetravalent) cation, *e.g.* $CeCl_3$ or $AlCl_3$, or if we wish to use salts with monovalent or bivalent cation the salts must be rendered sufficiently acid; *e.g.*, pH = 4.0 or less. Second, the collodion membrane must previously receive a treatment with gelatin. The gelatin will be rinsed out, but some gelatin obviously adheres to the wall. As I shall show in another paper this treatment

of the collodion membrane with a protein is necessary since otherwise water will diffuse through the collodion membrane in the form of positively charged particles and then the case is no longer comparable with that of gelatin-acid salts. This gelatin treatment of the collodion membrane is not required when we wish to experiment with positively charged particles of water, since water assumes practically always a positive charge when in contact with a collodion membrane free from gelatin, even in comparatively high concentrations of acid.

If we use membranes which had contained a 1 per cent gelatin solution over night but were then freed from the gelatin by a number of washings with warm water, and if we substitute for the gelatin-acid salt a crystalloidal salt which causes water to be charged negatively in such gelatin-treated membranes, *e.g.* M/512 $Al_2Cl_6$, we notice the same depressing effect of an increase of the concentration of electrolytes on the initial rate of diffusion of water into the solution as was described in the case of the solutions of gelatin-acid salts. When we separate a collodion bag filled with M/512 $Al_2Cl_6$ (which causes water to be charged negatively) and put it into $H_2O$, the level of liquid in the manometer will rise to a height of about 300 mm. in 20 minutes. When we add varying quantities of a neutral salt to the M/512 solution of $Al_2Cl_6$, and put the same concentration of salt into the outside beaker containing the water—in order to eliminate the direct effect of the salt added upon the rate of diffusion—we notice that the rate of diffusion of water will diminish the more rapidly the more salt we add. This is illustrated in Fig. 15. $NaNO_3$ and $CaCl_2$ have about the same depressing effect, showing that the depression is not due to the influence of the cation; while $SO_4$ and still more citrate depress much more powerfully than the chlorides, showing that the depression is due to the influence of the increasing concentration of the anion upon the negatively charged particles of water.

Similar experiments were made with solutions of $CaCl_2$ which were rendered sufficiently acid in order to cause the water to be charged negatively. If we select M/128 solutions of such acidulated $CaCl_2$ as a substitute for gelatin-acid solutions, we find that the addition of acid as well as of neutral salt causes a depression of the influence of

the acidulated $CaCl_2$ solution upon the initial rate of diffusion of water into the solution.

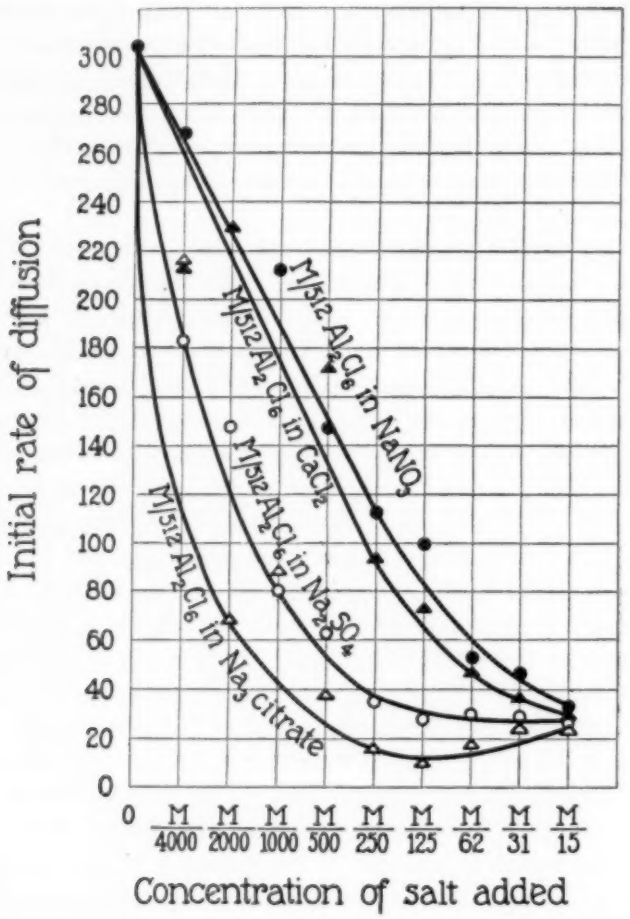



FIG. 15. M/512 $Al_2Cl_6$ separated from water by collodion bag. Depressing effect of addition of salt to M/512 $Al_2Cl_6$ upon the initial rate of diffusion of water into the bag. $SO_4$ depresses more than $NO_3$ or Cl.

In all the experiments mentioned the electrolyte was added not only to the inside but also to the outside solution. It is hardly necessary to state that the depressing effect is also well marked if the electrolyte is added only to the solution inside the collodion bag, while on the outside is distilled water.

These facts, then, show that the depressing effect of the addition of electrolyte upon the osmotic pressure of gelatin solutions (or colloidal solutions in general) is paralleled by the effect the addition of electrolyte has upon the initial rate of diffusion from water into solution through a collodion membrane, when we substitute a crystalloidal electrolyte for the gelatin salt.

## SUMMARY.

1. When a 1 per cent solution of a metal gelatinate, *e.g.* Na gelatinate, of pH = 8.4 is separated from distilled water by a collodion membrane, water will diffuse into the solution with a certain rate which can be measured by the rise of the level of the liquid in a manometer. When to such a solution alkali or neutral salt is added the initial rate with which water will diffuse into the solution is diminished and the more so the more alkali or salt is added. This depressing effect of the addition of alkali and neutral salt is greater when the cation of the electrolyte added is bivalent than when it is monovalent. This seems to indicate that the depressing effect is due to the cation of the electrolyte added.

2. When a neutral M/256 solution of a salt with monovalent cation (*e.g.* $Na_2SO_4$ or $K_4Fe(CN)_6$, etc.) is separated from distilled water by a collodion membrane, water will diffuse into the solution with a certain initial rate. When to such a solution alkali or neutral salt is added, the initial rate with which water will diffuse into the solution is diminished and the more so the more alkali or salt is added. The depressing effect of the addition of alkali or neutral salt is greater when the cation of the electrolyte added is bivalent than when it is monovalent. This seems to indicate that the depressing effect is due to the cation of the electrolyte added. The membranes used in these experiments were not treated with gelatin.

3. It can be shown that water diffuses through the collodion membrane in the form of positively charged particles under the conditions mentioned in (1) and (2). In the case of diffusion of water into a neutral solution of a salt with monovalent or bivalent cation the effect of the addition of electrolyte on the rate of diffusion can be explained on the basis of the influence of the ions on the electrifica-

tion and the rate of diffusion of electrified particles of water. Since the influence of the addition of electrolyte seems to be the same in the case of solutions of metal gelatinate, the question arises whether this influence of the addition of electrolyte cannot also be explained in the same way, and, if this be true, the further question can be raised whether this depressing effect necessarily depends upon the colloidal character of the gelatin solution, or whether we are not dealing in both cases with the same property of matter; namely, the influence of ions on the electrification and rate of diffusion of water through a membrane.

4. It can be shown that the curve representing the influence of the concentration of electrolyte on the initial rate of diffusion of water from solvent into the solution through the membrane is similar to the curve representing the permanent osmotic pressure of the gelatin solution. The question which has been raised in (3) should then apply also to the influence of the concentration of ions upon the osmotic pressure and perhaps other physical properties of gelatin which depend in a similar way upon the concentration of electrolyte added; *e.g.*, swelling.

5. When a 1 per cent solution of a gelatin-acid salt, *e.g.* gelatin chloride, of pH 3.4 is separated from distilled water by a collodion membrane, water will diffuse into the solution with a certain rate. When to such a solution acid or neutral salt is added—taking care in the latter case that the pH is not altered—the initial rate with which water will diffuse into the solution is diminished and the more so the more acid or salt is added. Water diffuses into a gelatin chloride solution through a collodion membrane in the form of negatively charged particles.

6. When we replace the gelatin-acid salt by a crystalloidal salt, which causes the water to diffuse through the collodion membrane in the form of negatively charged particles, *e.g.* M/512 $Al_2Cl_6$, we find that the addition of acid or of neutral salt will diminish the initial rate with which water diffuses into the M/512 solution of $Al_2Cl_6$, in a similar way as it does in the case of a solution of a gelatin-acid salt.

# QUANTITATIVE LAWS IN REGENERATION. I.

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Regeneration consists in the growth of resting cells or buds as a consequence of the removal of parts of the body. Two problems are, therefore, involved in this phenomenon; namely, first, the quantity of growth which is measured by the mass of the regenerated organs, and, second, the problem of inhibition, or the fact that regeneration or growth of the resting cells or buds does not take place without removal of part of the body.

## *I. The Law of Direct Proportionality between the Mass of Sister Leaves of Bryophyllum and the Mass of Roots and Shoots Produced by Them.*

The writer had already shown that equal masses of sister leaves of *Bryophyllum calycinum* when isolated from the rest of the plant produce in equal time equal masses of shoots under equal conditions of temperature, moisture, and illumination.[1] The following measurements show that the same law holds also for the production of roots. In order to be able to measure the mass of roots correctly, it was necessary to let the roots develop in water. Part of a leaf was dipped into water, and in the wetted notches shoots and roots grew out rapidly, thereby preventing the growth of roots and shoots from the non-submerged notches of the leaf. In order to get accurate measurements of root production it was necessary to determine the dry weight of the roots. In addition, the dry weight of the leaves and of the shoots was also determined. The experiments lasted usually from 4 to 5 weeks in order to get larger masses of roots and to diminish the error in determining the quantity of regeneration. The organs were dried in an oven at a temperature of between 100° and 110°C. for about 24 hours.

[1] Loeb, J., *Bot. Gaz.*, 1918, lxv, 150.

Five pairs of sister leaves were suspended sidewise, the lower edge dipping in water, and roots and shoots formed at the lower edges only. Table I gives the dry weights of the leaves, of the shoots, and of the roots at the end of the experiment. The figure in parentheses, behind the dry weight of the shoots, expresses the number of shoots formed. The first horizontal row gives the dry weights of these organs for one set of five leaves (Set I), the second horizontal row gives the dry weights of these organs for the five sister leaves (Set II). The third horizontal row gives the ratio of the dry weights of the two sets of organs. The masses of the two sets of sister leaves were almost alike, and the ratio of the two masses was 1.02. According to the law expressed in the writer's former publications the

TABLE I.

*Duration of Experiment 34 Days.*

| | Dry weight of | | |
|---|---|---|---|
| | Leaves. | Shoots. | Roots. |
| | gm. | gm. | gm. |
| Set I. | 1.755 | 0.247 (14) | 0.113 |
| " II. | 1.718 | 0.247 (15) | 0.120 |
| Ratio $\frac{\text{dry weight I}}{\text{dry weight II}}$ | 1.02 | 1.00 | 0.94 |

mass of shoots and of roots produced by the two sets of leaves should also be alike (within the limits of accuracy of these experiments). The ratio of the two masses of shoots and roots should, therefore, have been approximately 1.00.

The figures of the third row show that the ratio of the dry weights of the two sets of shoots is exactly 1.00 and the ratio of the two sets of roots is 0.94. *The two sets of sister leaves having equal mass produced, during the same time and under equal conditions of temperature, moisture, and illumination, equal masses of shoots and of roots.*

In the next experiment one set of six isolated leaves (Set II) remained intact while the mass of each leaf of the second set was reduced to approximately one-half by cutting away one side of each leaf (Set I) (Fig. 1). The masses of the two sets of leaves were

therefore no longer equal but had approximately the ratio of about 1:2. It was to be expected that the dry weights of the shoots and roots produced by the two sets of leaves should also be in the ratio of about 1:2, and this was the case (within the limits of the possible accuracy of such experiments) (Table II). The ratio of the dry weights of the two sets of leaves was 0.54, the ratio of the weights

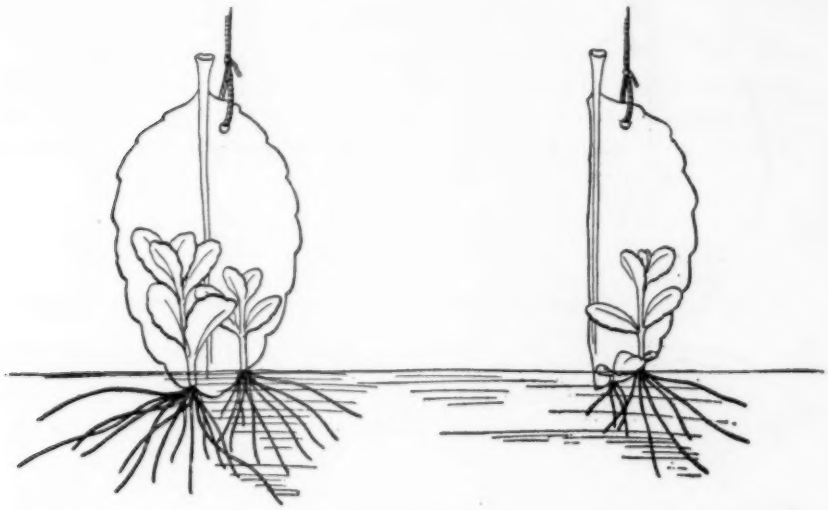

FIG. 1. Root and shoot formation in whole leaf and in sister leaf (from which the left half is cut off) dipping with their apices in water. Root and shoot formation only in the wetted parts of leaf. Diagrammatic.

TABLE II.

*Duration of Experiment 30 Days.*

| | Dry weight of | | |
|---|---|---|---|
| | Leaves. | Shoots. | Roots. |
| | *gm.* | *gm.* | *gm.* |
| Set I (six half leaves) | 1.245 | 0.174 (10) | 0.054 |
| " II (" whole " ) | 2.300 | 0.283 (16) | 0.092 |
| Ratio $\frac{\text{dry weight I}}{\text{dry weight II}}$ | 0.54 | 0.61 | 0.59 |

of shoots produced by them was 0.61, and the ratio of roots produced was 0.59. The tips of the two sets of leaves dipped in water and roots and shoots developed only in the submerged notches (Fig. 1).

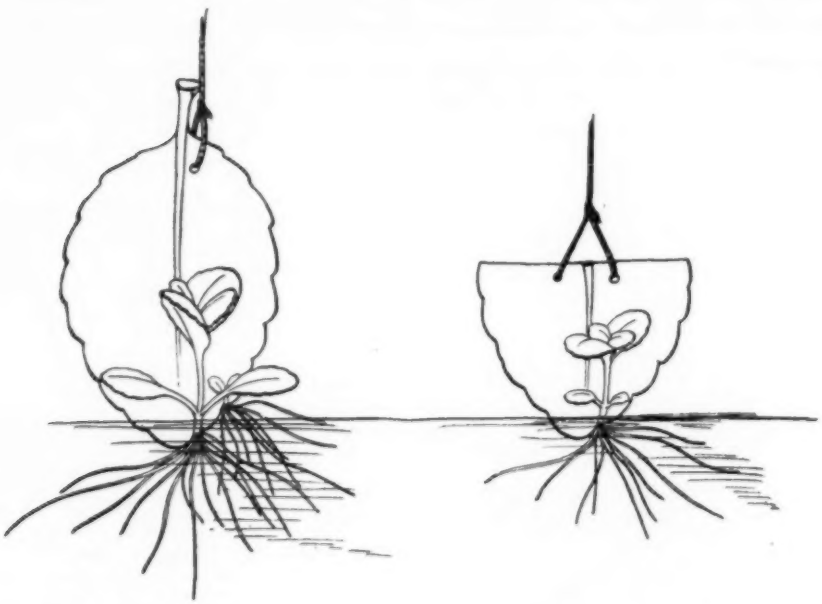

FIG. 2. Shoot and root formation in whole leaf and in sister leaf (from which the basal part is cut off), both dipping with their apices in water. Duration of experiment 34 days. After nature.

TABLE III.

*Duration of Experiment 33 Days.*

| | Dry weight of | | |
|---|---|---|---|
| | Leaves. | Shoots. | Roots. |
| | gm. | gm. | gm. |
| Set I (six reduced leaves)..... | 0.794 | 0.156 (11) | 0.043 |
| " II (" whole " )..... | 2.127 | 0.343 (19) | 0.116 |
| Ratio $\frac{\text{dry weight I}}{\text{dry weight II}}$..... | 0.37 | 0.45 | 0.37 |

In the next experiment one set of six leaves remained intact (Set II) while more than half of the basal part of the set of sister leaves (Set I) was cut off (Fig. 2). The tips of the leaves dipped into water. Again the ratio of the mass of shoots and roots regenerated was, within the limits of the accuracy of the experiments, the same as the ratio of the masses of the two sets of leaves. It was 0.37 for the leaves and exactly the same number for the roots, while it was slightly larger, 0.45, for the shoots (Table III).

In the next set, the leaves were suspended sidewise, their lower edges dipping in water (Fig. 3). In both sets of leaves the upper edge was cut off; in one set the lower edge with the exception of one

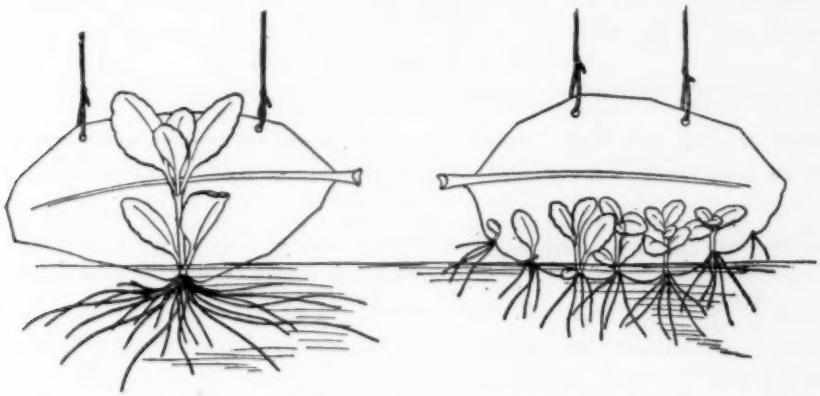

FIG. 3. Leaves suspended sidewise. The upper edge is cut off in both leaves. In leaf to left all notches except one removed, in leaf to right all notches on the lower side preserved. Duration of experiment 34 days. After nature.

or two notches was cut off (Set I), while in the other set (Set II) all the notches of the lower edge were left intact. This was done to make the number of shoots produced by both sets unequal and to show that the general law enunciated is independent of the number of shoots produced. Thus, as Table IV shows, the leaves of Set I produced six and the leaves of Set II produced sixteen shoots, yet the mass of the two sets of shoots was almost the same, though it is not impossible that when more shoots develop in one leaf the available material furnished by the leaf can be utilized more fully than when all the material goes into one shoot.

On the basis of these and of the writer's earlier experiments we can enunciate the following law.

Equal masses of sister leaves of *Bryophyllum calycinum* produce in equal time, under equal conditions of temperature, moisture, illumination, and aeration, approximately equal masses of roots and shoots, regardless of the number of shoots or roots formed (except that a moderate number of shoots may possibly permit a more complete utilization of the material furnished by the leaf than if only one shoot is formed). When the masses of two sister leaves are unequal, the masses of shoots and roots produced by them are directly proportional to the masses of the leaves.

TABLE IV.

*Duration of Experiment 33 Days.*

| | Dry weight of | | |
|---|---|---|---|
| | Leaves. | Shoots. | Roots. |
| | gm. | gm. | gm. |
| Set I. Five leaves with one or two notches..... | 1.810 | 0.248 (6) | 0.106 |
| " II. " sister leaves with more " ..... | 1.778 | 0.270 (16) | 0.121 |
| Ratio $\frac{\text{dry weight I}}{\text{dry weight II}}$ ..... | 1.02 | 0.92 | 0.88 |

## *II. Ratio of the Mass of Shoots to the Mass of Roots Produced by a Leaf Dipped in Water.*

When we dip a leaf with part of its edge into water, shoots and roots form as a rule only in the wetted part of the leaf. It was of importance to determine the ratio of dry weight of roots to the dry weight of shoots produced in such cases. In the examples already quoted this ratio was never less than one-third and never more than one-half. The average taken from a large number of experiments gave the ratio of the dry weight of roots to that of shoots under such conditions as 0.42. This figure will be used in the experiments of the next chapter.

### *III. The Inhibitory Influence of the Stem on Shoot Production in the Leaf of Bryophyllum calycinum.*

Only the leaf isolated from the stem is capable of forming shoots; it suffices as a rule to leave a piece of stem connected with the leaf to cause retardation or inhibition of the growth of the dormant buds in the notches of a leaf.[2] There are two possible reasons for the inhibitory action of the stem on the shoot and root formation in the leaf. The material available for root and shoot formation in the leaf either flows naturally into the stem and hence the buds in the notches of the leaf cannot grow out on account of lack of material for growth;[3] or second, the stem sends into the leaf a substance preventing the growth of the notches in spite of the fact that the material needed for the growth of the dormant buds in the notches of the leaf is available.

Only quantitative experiments allow us to decide between the two possibilities. If the assumption is correct that the leaf normally sends the material which can be utilized for the growth of shoots and roots into the stem, the stem in connection with a leaf should gain in weight and this gain should be equal to the mass of shoots and roots the same leaf would produce when separated from the stem. If the other possibility is correct, and if we are dealing with the effect of inhibitory substances sent into the leaf, no such increase in the dry weight of the stem need occur. My experiments give a clear answer in favor of the first possibility; namely, that the inhibitory effect of the stem upon the shoot and root production in the leaf is due to the fact that the material in the leaf which could be utilized for shoot and root formation flows normally into the stem, as long as the leaf is connected with the stem and the sap flow is not interrupted; while when the leaf is separated this material becomes available for the growth of shoots and roots in the leaf, and the inhibition ceases. Of course, we cannot determine directly which mass of shoots and roots the inhibited leaf would have produced if

[2] This inhibition cannot be permanent since before the falling off of the leaf the sap flow between leaf and stem will cease and this will have the same effect as the cutting off of the leaf.

[3] Loeb, J., *Ann. Inst. Pasteur*, 1918, xxxii, 1.

its regeneration had not been inhibited by the stem, but we can determine this quantity indirectly, by measuring the quantity of shoots and roots produced by the sister leaf detached from the stem, since we know that two sister leaves of equal size produce equal masses of shoots and roots in equal time and under equal conditions.

Our method of procedure was as follows. Pieces of stem possessing one node with two healthy leaves of equal size were cut out (Fig. 4). The stem was divided lengthwise as accurately as possible into two equal pieces, $b$ and $b_1$, by a cut between the base of the petioles of the two leaves, so that each leaf was connected with one-half piece of stem of equal mass. One leaf ($a_1$) remained in connection with its piece of half stem ($b_1$), while the other leaf ($a$) was detached from its piece ($b$). The dry weight of this detached piece of half stem $b$ was determined immediately at the beginning of the experiment.

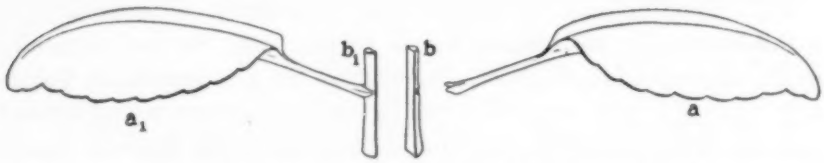


FIG. 4. Diagram to illustrate experiments in which the stem is cut lengthwise.

At the end of the experiment the other piece of half stem $b_1$ was detached from its leaf and its dry weight was determined. By comparing the dry weight of $b_1$ with that of $b$ (detached at the beginning of the experiment) it was possible to ascertain how much material the piece $b_1$ had received from the leaf during the time the experiment lasted. Both leaves, the one detached from the stem as well as the one left in connection with the stem, were suspended in a moist aquarium, the apex of each leaf dipping in water. The detached leaves formed shoots and roots very rapidly, while the leaves in connection with their half pieces of stem formed practically no shoots and roots.[4]

[4] The inhibitory power of a piece of stem on the shoot formation in the leaf increased within certain limits with the mass of the stem, but not in direct proportion. It was also very obvious that equal masses of stem suppressed the

We selected for each experiment twelve pairs of sister leaves and care was taken that the leaves were healthy, not too large, and of equal size. We made sure that the fresh weight of each set of leaves with their piece of half stem was approximately like that of the set of sister leaves (Table V).

The difference in one set is not more than 3 per cent and is equalized by the fact that six sets of different experiments were made. We proceeded then as follows. The dry weight of $b$ (Fig. 4) was ascertained immediately. Then the leaf $a$ (detached from its stem) and the leaf $a_1$ (in connection with its piece of half stem) were suspended in the same aquarium dipping with their apices into water. In the leaves $a$ (without stems), roots and shoots formed soon, while none, or practically none, formed in leaves $a_1$.

TABLE V.

| No. of experiment. | Fresh weight of | |
|---|---|---|
| | $a+b$ | $a_1+b_1$ |
| | gm. | gm. |
| 1 | 28.240 | 29.103 |
| 2 | 36.625 | 37.175 |
| 3 | 40.930 | 39.640 |

After several weeks the shoots were cut off, their dry weight was determined, and the piece of stem $b_1$ was also detached from its leaf and its dry weight determined. Let $a$ be the dry weight of shoots and roots formed by the leaves detached from their stem; let $a_1$ be the dry weight of shoots and roots formed by the leaves or in the axil of the leaves connected with their stem; let $b$ be the dry weight

shoot formation the more completely the smaller the leaf. When the leaf is large it furnishes more material than the cells of a small piece of stem can absorb. It is also of interest that the inhibitory power of a piece of stem on shoot formation in a leaf is smaller in an old leaf than in a young leaf nearer the apex. This influence of age of the leaf is probably connected with the fact that in the oldest leaf the flow of sap into the leaf is more or less incomplete; this interruption of sap flow may possibly be the first step in the histological changes at the base of the leaf which cause it to fall off. In our experiments conditions were selected in such a way as to make the inhibition of shoot and root formation in Leaf $a_1$ practically complete.

of the piece of half stem at the beginning, $b_1$ the dry weight of the piece of half stem at the end; then we should expect the following relation to hold (within the limits of accuracy of the experiments).

$$a - a_1 = b_1 - b$$

Six sets of experiments (each with twelve pairs of leaves) were made in which the dry weights of the shoots and of the pieces of stem were determined. The stems were either 2, 4, or 8 cm. long, and with this length of stem the formation of shoots on the pieces connected with the stem was almost completely suppressed. The weights of the roots were not determined but we know that on the average the dry weight of the roots formed by the leaves under the

TABLE VI.

| No. of experiment. | Duration of experiment. | $a$ | $a_1 + b_1$ | $b$ | $a_1 + b_1 - b$ |
|---|---|---|---|---|---|
| | *days* | *gm.* | *gm.* | *gm.* | *gm.* |
| 1 | 28 | 0.141 | 0.384 | 0.176 | 0.208 |
| 2 | 28 | 0.172 | 1.1095 | 0.825 | 0.284 |
| 3 | 28 | 0.308 | 0.7348 | 0.3846 | 0.350 |
| 4 | 29 | 0.216 | 0.496 | 0.159 | 0.337 |
| 5 | 29 | 0.166 | 1.146 | 0.829 | 0.317 |
| 6 | 29 | 0.267 | 0.759 | 0.364 | 0.395 |
| Total..... | | 1.270 | | | 1.891 |

conditions of our experiments is 42 per cent of the dry weight of the shoots formed. Hence we must add this value in our final results to $a$ (but not to $a_1$, since practically no shoots or roots were formed in the leaves connected with the stems). Table VI gives the dry weight determinations for the six sets of experiments. The dry weight of the shoots produced by all the detached leaves in the six sets of experiments is 1.270 gm. To this value must be added the dry weight of the roots produced which amounts to 42 per cent of the dry weights of shoots; namely, 0.533 gm. The value for $a$ then is $1.270 + 0.533 = 1.803$ gm.

The value for $a_1 + b_1 - b$ was equal to 1.891 gm. Practically no shoots or roots were produced by these leaves connected with their stems, so that no correction for roots is necessary. Hence the value

1.891 represents the gain in mass of the stem. We therefore find that $a = 1.803$, and that $a_1 + b_1 - b = 1.891$. The ratio of $\frac{a}{a_1 + b_1 - b}$ should be = 1. In our experiments it is = 0.95, which is as near the theoretical value as the accuracy of our experiments permits.

We, therefore, can state: When a leaf is connected with a piece of stem the stem gains in weight and this gain equals the weight of roots and of shoots formed by the sister leaves detached from the stem. The inhibitory effect of the stem upon shoot and root formation by the leaf is therefore adequately explained by these figures as being due to the fact that the material available for growth normally flows from the leaf into the stem.

## SUMMARY.

1. Equal masses of sister leaves of *Bryophyllum calycinum* produce equal masses of shoots and roots in equal time and under equal conditions.

2. The mass of shoots and roots produced by different masses of sister leaves in equal time and under equal conditions is approximately in direct proportion to the masses of the leaves.

3. When a piece of stem inhibits the production of shoots and roots in a leaf of *Bryophyllum* connected with it, the stem gains in mass and this gain in mass equals approximately the mass of shoots and roots the leaf would have produced if it had been detached from the stem.

4. This suggests that the inhibitory influence of the stem upon the formation of shoots and roots in the leaf is due to the fact that the material available for this process naturally flows into the stem.